ASTROCYTES IN PSYCHOTIC DISORDER

by

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Abstract

Astrocyte dysregulation has been implicated in the pathophysiology of schizophrenia (SCZ) and bipolar disorder (BPD), however the exact nature of astrocytic alterations remains to be identified. I investigated whether levels of four astrocyte-specific proteins; glial fibrillary acidic protein (GFAP), aldehyde dehydrogenase type 1L1 (ALDH1L1), vimentin, and excitatory amino acid transporter type 1 (EAAT1) are altered in SCZ and BPD. Immunohistochemical staining of ALDH1L1 and GFAP in human grey and white matter was also performed, and staining patterns compared qualitatively. Relative concentrations of GFAP, ALDH1L1, vimentin, and EAAT1 were assessed post-mortem in the dorsolateral prefrontal cortex in SCZ (n=35), BPD (n=34) and non-psychiatric control (n=35) groups by western blotting. The same proteins were also quantified in the cingulate cortex of rats administered the antipsychotics haloperidol and clozapine. Elevated levels of GFAP were observed in SCZ and BPD, when compared to controls. GFAP was also significantly increased in individuals with psychotic symptoms, when compared to those without. Vimentin, ALDH1L1 and EAAT1 levels did not differ between groups. Rats exposed to antipsychotics did not exhibit significant overall differences in any astrocytic protein, suggesting that increased GFAP in SCZ is not attributable to antipsychotic treatment. Our findings indicate that astrocyte pathology may be associated with psychotic symptoms. Lack of ALDH1L1 and vimentin variability, paired with increased GFAP levels, may imply that astrocyte numbers are unchanged but astrocytes are partially activated, or may indicate a specific dysregulation of GFAP. Immunohistochemical results suggest that ALDH1L1 may be a more reliable marker of astrocytes than GFAP in human grey matter.

Preface

All material presented in this dissertation, with the exception of immunohistochemical data presented in section 3.3 and discussed in section 4.5, has been published as Feresten, A. H., Barakauskas, V., Ypsilanti, A., Barr, A. M., & Beasley, C. L. (2013). Increased expression of glial fibrillary acidic protein in prefrontal cortex in psychotic illness. Schizophrenia Research, 150(1)252-257.

Figure 2 is used with permission from Barakauskas, V. E., Ypsilanti, A. R., Barr, A. M., Innis, S. M., Honer, W. G., & Beasley, C. L. (2010). Effects of sub-chronic clozapine and haloperidol administration on brain lipid levels. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 34(4)669-673.

Treatment of rats and preparation of rat tissue was performed by Vilte Barakauskas, Athena Ypsilanti, Dr. Clare Beasley and Dr. Alasdair Barr. The Stanley Foundation Neuropathology Consortium in Bethesda, Maryland, USA collected and dissected all human tissue. I performed all protein analysis and immunohistochemistry. Dr. Beasley and I conceived of the experiments and I wrote the manuscript for the published paper, with substantial input from Dr. Beasley.

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List of abbreviations

ACC; Anterior cingulate cortex

ALDH1L1; Aldehyde dehydrogenase type 1L1

AMPA; α -amino-3-hydroxy-5-methyl-4-isoxazoleprorionic acid

BDNF; Brain-derived neurotrophic factor

BPD; Bipolar Disorder

CNS; Central nervous system

CRH; Corticotropin releasing hormone

DISC1; Disrupted in schizophrenia 1

DLPFC; Dorsolateral prefrontal cortex

DSM; Diagnostic and Statistical Manual of Mental Disorders

EAAT1/2; Excitatory amino acid transporter type 1/2

EPS; Extrapyramidal syndrome

FDH; 10-formyltetrahydrofolate dehydrogenase

GABA; Gamma-aminobutyric acid

GAD; Glutamic acid decarboxylase

GDNF; Glial-derived neurotrophic factor

GFAP; Glial fibrillary acidic protein

GLAST; Glutamate aspartate transporter

MDD; Major Depressive Disorder

NFkB; Nuclear factor kappa-light-chain-enhancer of activated B cells

NMDA; N-methyl-D-aspartate

NMS; Neuroleptic malignant syndrome

NO; Nitrous oxide

PCP; Phencyclindine

PFC; Prefrontal cortex

PMI; Post mortem interval

QKI; Quaking

SCZ; Schizophrenia

TNFα; Tumor necrosis factor alpha

TUNEL; Terminal deoxynucleotidyl transferase dUTP nick end labeling

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1. Introduction

1.1. Schizophrenia

Schizophrenia (SCZ) is a devastating psychiatric disorder affecting 0.5-1% of the population globally **(**McGrath et al, 2008). SCZ is typified by the presence of psychosis, as well as negative symptoms. For DSM-IV-TR criteria **(**American Psychiatric Association, 2000) see Appendix 1. Psychosis is defined by hallucinations (sensory experience independent of sensory input), delusions (fixed ideation based on incorrect interpretation of a real or imagined event), and/or disorganization of thoughts and behavior (including but not limited to; word salad, stereotyped behavior, catatonia). The term "negative symptoms" indicates a loss or decrease in expected behavior, such as; anhedonia, flat affect, poverty of speech, avolition, and asociality. Cognitive symptoms have also been associated with SCZ, including working memory and attentional deficits, as well as difficulties with executive function (Gold and Harvey, 1993)

1.1.1. Epidemiology

Disease onset occurs most commonly during adolescence, with women developing symptoms a couple years later than men on average **(**Hafner et al. 1993**)**. Women also have an increased risk of developing SCZ later in adulthood (Hafner et al. 1993; Leung and Chue, 2000). The etiology of SCZ remains unknown, however it is becoming clear that complex genetic and environmental factors are involved in the development of this disorder. SCZ is approximately 73-90% heritable, with a 50% concordance rate between identical twins (Sullivan et al. 2003), implying that while genetics play a large role in susceptibility to the disorder, environmental factors also influence disease development.

Research into potential environmental contributions to this disorder has implicated a wide range of factors including prenatal infection (Brown and Derkits, 2010), cannabinoid exposure (Arseneault et al. 2004), and urbanicity (Krabbendam and Van Os, 2005).

1.1.2. Treatment

SCZ is treated primarily with medication, although psychotherapeutic approaches may also be used. Antipsychotic drugs are the most common treatment for SCZ. The first antipsychotic drugs, Chlorpromazine and subsequently Haloperidol, were discovered in the 1950s and later shown to act through blockade of the dopamine receptor D2 (Curzon, 1990). These first generation, or "typical," antipsychotics are somewhat effective at acutely reducing positive symptoms of SCZ, but have little effect on negative or cognitive symptoms. They also frequently cause extrapyramidal syndrome (EPS), a cluster of movement disorders including dystonia, dyskinesia, pseudoparkinsonism, and akathisia, caused by extended blockade of dopamine receptors in the basal ganglia (Farde et al. 1992). Long-term exposure to high doses of typical antipsychotics can also lead to the development of tardive dyskinesia.

Clozapine, the first of the second generation, or "atypical," antipsychotics, was first developed in the 1960s but due to it's potential to induce agranulocytosis, it fell off the pharmaceutical radar. However, in 1988, Kane and colleagues demonstrated its efficacy in improving symptoms of treatment-resistant psychosis (Kane et al. 1988). This sparked an exploration into atypical antipsychotic development, producing several other atypical drugs including Olanzipine (Zyprexa), Risperidone (Risperdal), and Quetiapine (Seroquel). Atypical antipsychotics may be more effective at treating negative symptoms

of SCZ than are typical antipsychotics, and are less likely to cause EPS or tardive dyskinesia. However they can produce metabolic side effects including dramatic weight gain and increased risk for diabetes and cardiovascular disease (Haddad and Sharma, 2007), and the risk of agranulocytosis associated with Clozapine and other related atypical antipsychotics necessitates regular blood tests.

1.2. Bipolar disorder

Bipolar disorder (BPD) is another distressing disorder effecting about 1% of the global population (Merikangas et al. 2011). BPD is typified by episodes of extreme mood disturbances. The DSM-IV-TR recognizes two subtypes of bipolar disorder (DSM-IV-TR, American Psychiatric Association, 2000). BPD II is defined by one or more major depressive episodes accompanied by at least one hypomanic episode. BPD I is the more severe form, characterized by at least one manic or mixed episode. For diagnostic criteria for BPD see Appendix 1.

A major depressive episode is a period of two consecutive weeks, during which the patient continuously experiences a series of symptoms including but not limited to; depressed mood, loss of energy, and thoughts of worthlessness. A hypomanic episode is a period of four or more days during which the patient experiences an elevated or irritable mood, accompanied by several other symptoms such as; thoughts of grandiosity, decreased need for sleep, hedonism, and increased risk-taking. Symptoms associated with hypomania are not severe enough to interfere with social functioning or necessitate hospitalization, and psychosis is not evident. Mania is a more severe form of hypomania, in which symptoms last for at least a full week and result in pronounced social and/or

functional disturbances that may require hospitalization. A mixed mood episode is a period of at least one week during which the patient experiences symptoms fulfilling the requirements of both a major depressive and a manic episode.

Psychosis may occur in both mixed and manic episodes, and is present in at least half of all patients with BPD I (Keck et al. 2003**)**. While not part of the DSM-IV-TR description of BPD, recent research has also uncovered cognitive deficits associated with BPD that resemble those observed in SCZ. It is unclear whether these symptoms are specifically associated with psychosis in BPD, as suggested by Simonsen and colleagues (2011), or if they increase with disease severity, as indicated in two meta-analyses performed by Bora and colleagues (2010; 2011).

1.2.1. Epidemiology

As in SCZ, BPD onset occurs most commonly during adolescence and etiology remains unknown. Studies suggest heritability of BPD to be 60-85% (Smoller and Finn, 2003), giving genetics a large role to play in this disorder than seen in SCZ. However, investigations into genetic risk factors for BPD have failed to produce any clear picture of a genetic profile for this illness, suggesting a complex system of heritability (Sklar et al. 2008; Barnett and Smoller, 2009). Environmental factors also contribute to BPD etiology, specifically those associated with highly stressful life events (Johnson, 2005; Horesh et al. 2011).

1.2.2. Treatment

BPD is primarily treated with medication, sometimes with adjunct psychotherapeutic treatment. Electroconvulsive treatment may also be used in cases of extreme refractory depression or suicidality, and recent research into deep brain stimulation has shown some promise (Holtzheimer et al. 2012). The most common medications for BPD are lithium carbonate and anticonvulsants (including valproate).

The use of lithium salts as a mood-stabilizing agent has a long and rich history, dating back at least to the 19th century, and possibly as early as ancient Greece (Kline, 1973). The pathways through which lithium affects BPD symptoms remain unclear. Lithium ions influence many different neurotransmitter systems, including serotonin and dopamine (Price et al. 1990; Lenox and Hahn, 2000), and also regulate circadian rhythms (McQuillin et al. 2007) and the release of neurotrophic factors (Machado-Vieira et al. 2009). Valproate is also widely used to treat BPD. It is an anticonvulsant developed in the late 19th century and recognized for it's anti-manic properties in the 1960s (Pope et al. 1991). The mechanisms of action for valproate and other anticonvulsants in the treatment of BPD also remain unclear.

1.3. Comorbidities

People with SCZ and BPD appear to be more prone to a shocking number of illnesses (Carney et al. 2006; Carney and Jones, 2006), especially cardiovascular disease (Birkenaes et al, 2007), and substance abuse (Ringen et al. 2008). SCZ appears to increase risk for cardiovascular disease and diabetes independent of the metabolic symptoms associated with exposure to atypical antipsychotics (Haddad and Sharma et al.

2007). It has also been suggested that poor hygiene and nutrition contribute to the increased rate of chronic illness seen in patients with SCZ, resulting in a 20-year decrease in life expectancy (Tiihonen et al, 2009). BPD is associated with a 10-year decrease in life expectancy (Chang et al. 2011), and an increase in chronic illness similar in breadth to that seen in SCZ, but at lower rates. Risk of suicide is also twice as common in individuals with SCZ and BPD, and this likely contributes to the increased mortality rates associated with these disorders (Laursen et al. 2007).

People with SCZ and BPD are about twice as likely to use illicit drugs and alcohol as their unaffected counterparts (Regier et al. 1990; Ringen et al. 2008). Furthermore, between 51% and 74% of people with psychotic disorders are also smokers (Ringen et al. 2008; Diaz et al, 2009). As the dopamine system is affected in both these disorders, it is possible that the mesolimbic reward circuit responsible for addiction is altered, increasing risk for addictive behavior (Krystal et al. 2006).

1.4. Schizophrenia and bipolar disorder: dichotomy

In addition to overlapping symptoms, SCZ and BPD share genetic and environmental risk factors, leading many authors to suggest that these disorders may have common etiologies. While it has been shown that SCZ and BPD are both highly heritable (Levinson and Mowry, 1999; Craddock and Jones, 1999), a recent large scale study in Switzerland involving over two million family pedigrees showed that risk for both disorders is increased in families with either SCZ or BPD (Lichtenstein et al, 2009). Furthermore, while genes have been identified that increase risk for specifically SCZ or specifically BPD, many genes have been identified that increase risk for either diagnosis,

suggesting a highly complex and overlapping genetic profile for these disorders (Berrettini, 2003). Craddock and colleagues discuss this phenomenon at length in their 2009 and 2013 publications (Craddock et al. 2009; Craddock and Sklar, 2013).

Much discussion has been published in the past decade on the continued viability of the Kraepelinian dichotomy; the concept proposed by Emil Kraepelin in 1899 that SCZ and BPD are distinct and non-overlapping syndromes (Jablensky, 2007). Despite criticism and building evidence against this theory, it continues to define current diagnostic practices used by psychiatrists, and thus by researchers. Other systems that have been proposed include spectrum (Linscott and Van Os, 2010), hierarchical (Murray et al. 2004; Kamaz and Van Os, 2009), and multi-dimentional (Craddock et al. 2009) diagnostic systems. However, while scientific consensus continues to migrate away from a diagnostic dichotomy between SCZ and BPD, the tools available to researchers (mainly DSM-IV-TR/DSM-5 and ICD-10) largely preclude study of psychiatric populations using these proposed classification systems.

1.5. DLPFC and psychosis

The dorsolateral prefrontal cortex (DLPFC; Brodmann's areas 9 and 46) is a region of the prefrontal cortex (PFC) involved in working memory, memory retrieval, and task analysis, among other top-down processing tasks (Van Snellenberg et al. 2009), and has been heavily implicated in the cognitive deficits associated with SCZ (Karlsgodt et al. 2007; 2009; Hamilton et al. 2009; see Volk and Lewis, 2010 for discussion). Recent work in BPD has identified similar cognitive deficits, also associated DLPFC dysfunction (Robinson et al, 2006; Lagopoulos et al. 2007; Haldane et al. 2008), and specifically in

BPD with psychosis (Simonsen et al. 2011; Anticevic et al. 2013). The DLPFC has also been implicated in other aspects of psychotic disorder. Connectivity between the DLPFC and the temporal cortex was found to be decreased in SCZ and negatively correlated with auditory hallucination severity (Lawrie et al. 2002), while DLPFC deficits have been correlated with avolition and anhedonia (Heerey et al. 2008). Combined, current evidence points towards the DLPFC as a particularly interesting structure in the context of psychotic illness and, in light of these finding, we chose to study this region.

1.6. Pathology of SCZ and BPD

While the pathophysiology of SCZ and BPD has not yet been identified, several theories have emerged. Developmental, neurotransmitter, and inflammatory dysregulation have all been documented in SCZ and BPD (e.g. Drexhage et al. 2010; Pennington et al. 2008a; Rao et al. 2013). In addition, it has been suggested that these disorders may have an ongoing progressive component (Pantelis et al. 2005). These theories are discussed in greater detail below.

1.6.1. Developmental hypotheses

The neurodevelopmental hypothesis of SCZ was formulated by authors Weinberger (1986; 1996; 2007), and Murray & Lewis (1987), and proposes that disruptions during brain development underlie the later emergence of SCZ during adulthood. Altered brain development has also been implicated in BPD. Evidence for this theory comes from several avenues.

People with SCZ, and to a lesser extent BPD, have increased incidence of pre- and peri-natal complications including; winter-spring birth, maternal infection, and obstetrical problems (Torrey et al. 1997; Buka and Fan, 1999; Lewis and Levitt, 2002; Yolken et al. 2006; Sanches et al. 2008). This observation suggests that these disorders may be associated with developmental perturbations early in life. Such events coinciding with early development could affect brain structure in ways that become most apparent after further remodeling, such as the frontal cortical remodeling that occurs during adolescence, when both of these disorders most commonly develop. This developmental hypothesis is also supported by somewhat controversial results suggesting altered neuronal migration during early development (Jakob and Beckmann, 1986; Kovelman and Scheibel, 1984; Akbarian et al. 1993), and altered Reelin levels **(**Guidotti et al. 2000), although these findings have proved hard to replicate (Lewis and Levitt, 2002). Developmental changes could also occur later, during childhood or adolescence. In SCZ, and to a lesser extent in BPD, authors have identified premorbid neurobehavioral abnormalities in childhood such as difficulties with information processing (Kent and Craddock, 2003; Stefanopoulou et al. 2009). These childhood symptoms are more prevalent in instances of early onset, and seem to correlate with disease severity and poor prognosis.

Furthermore, people with BPD have a dramatically increased rate of childhood trauma (Garno et al. 2005**;** Kauer-Sant'Anna et al. 2007). This observation has led authors to investigate the role of stress hormones and the hypothalamic pituitary axis in this disorder. Elevations in baseline serum cortisol levels have been shown in BPD (Cervantes et al. 2001), and patients in remission from BPD have increased cortisol release in response to a combined dexamethasone suppression/ corticotropin-releasing

hormone (CRH) challenge (Watson et al. 2004). Other authors have also found increased or altered cortisol activity in BPD regardless of current symptom expression (see Bond and Young, 2007). Cortisol directly modulates brain-derived neurotrophic factor (BDNF) production and function, which is also altered in BPD (Lin, 2009). BDNF plays a large role in neural and synaptic development, so BDNF imbalance could theoretically lead to a host of structural and functional abnormalities that could influence pathogenesis (Kapczinski et al. 2008).

Finally, in SCZ, it has been suggested that increased synaptic pruning in early development could lead to excessive synaptic remodeling in adolescence, precipitating illness (Hoffman and McGlashen, 1997**;** McGlashan and Hoffman, 2000**;** Lewis and Levitt, 2002). One suggestion is that this developmental over-pruning could be the result of glutamatergic N-methyl-D-aspartate (NMDA) receptor hypofunction, decreasing the rate with which silent synapses are activated in early development (du Bois and Huang, 2007). This idea is further supported by the findings that genes DISC1 (disrupted in schizophrenia 1) and neuregulin 1, two genes heavily implicated in SCZ, are important factors in cortical and synaptic development (Mei and Xiong, 2008; Duan et al. 2007**;** Jaaro-Peled et al. 2009). Interestingly, mutations in DISC1 and neuregulin 1 are also risk factors for BPD (Maeda et al. 2006; Thomson et al. 2006; Blackwood et al. 2007**)**, supporting a theory of overlapping genetic predisposition to both disorders that affects neurodevelopment early in life (Kaymaz and Van Os, 2010).

1.6.2. Neurotransmission

Neurotransmitter dysfunction has been heavily implicated in both SCZ and BPD. As antipsychotic drugs directly affect dopamine neurotransmission, SCZ was originally believed to be primarily a dopaminergic disorder, and several dopamine-based hypotheses were proposed (Snyder, 1976; Davis et al. 1991). These theories suggested that schizophrenia resulted from increased dopamine neurotransmission. However, these early dopamine theories failed to sufficiently account for symptoms of SCZ not associated with psychosis **(**Howes and Kapur, 2009**)**. More recently, several authors have popularized a more complex theory in which dopamine dysregulation in various brain regions is caused by imbalances of other neurotransmitters, in particular glutamate and gamma-aminobutyric acid (GABA) (Grace, 1991; Laruelle et al. 2003; Stone et al. 2007; Lisman et al. 2008). While dopamine agonists, such as amphetamines, are capable of eliciting positive-like symptoms in healthy test subjects, antagonists at glutamate NMDA receptors, such as phencyclindine (PCP), can elicit both positive- and negative-like symptoms (Malhotra et al. 1996). In SCZ there is substantial support for altered glutamate neurotransmission, and in particular NMDA receptor dysregulation (Stone et al. 2007; Lisman et al. 2008). NMDA receptors play a vital role in synaptic development, and are expressed on a subset of inhibitory interneurons that provide negative feedback to cortical pyramidal cells. Without this negative feedback, increased cortical glutamatergic output would result in increased dopaminergic activity in the prefrontal cortex (Lisman et al. 2008). Interestingly, cholinergic input from the medial septal region stimulates GABA release from cortical interneurons, inhibiting glutamatergic activity and possibly explaining why approximately 70% of patients with SCZ use nicotine. Nicotine activates

nicotinic acetylcholine receptors and thus increases cholinergic activity (Masterson and O'Shea, 1984; Dalack et al; 1998**;** Martin et al. 2004).

Glutamate abnormalities have also been observed in BPD (Michael et al. 2003), including altered expression of α-amino-3-hydroxy-5-methyl-4-isoxazoleprorionic acid (AMPA) (Beneyto and Meador-Woodruff, 2006) and NMDA receptors (McCullumsmith et al, 2007; Scarr et al. 2003). Of particular interest are observations of increased glutamate/glutamine ratios in frontal cortical regions of post mortem BPD subjects (Hashimoto et al. 2007; Lan et al. 2008), potentially indicating a hyperdopaminergic state in the BPD frontal cortex. This, paired with findings of altered glutamate transporter levels (Eastwood and Harrison, 2010**),** suggests altered presynaptic glutamate function in BPD (Chen et al. 2010*)*.

1.6.3. Inflammation

Abnormal expression of inflammatory factors has been reported in SCZ (Watanabe et al. 2010), and to a lesser extent in BPD (Berk et al. 2011). Developmental exposure to pathogens increases risk for SCZ over 3-fold (Brown et al. 2001; 2004), and increased psychosis-like behavior has been observed in rodents exposed to pathogens *in-utero* (Kneeland and Fatemi, 2013). Some people with SCZ express altered levels of cytokines (Potvin et al. 2008), with this observation leading to what is referred to as the cytokine hypothesis of SCZ. This theory proposes that exposure to maternal immune response *in-utero* alters neural and immune development, resulting in the development of pathology later on (Watanabe et al. 2010).

Increased plasma levels of pro-inflammatory cytokines in BPD, as well as the anti-inflammatory effects of mood stabilizers, have led authors to suggest a role of inflammation in BPD pathogenesis (Goldstein et al. 2009; Berk et al. 2011). Furthermore, BPD is highly co-morbid with chronic inflammatory diseases (Leboyer et al. 2012), strongly implicating inflammatory processes in the progression of the disorder. It has been suggested by Müller and colleagues that increased inflammation could lead to dysregulation of glutamate receptor systems that could further influence GABA and dopamine systems (Lisman and colleagues, 2008) and that these observations could be behind the disease processes of both affective and psychotic illnesses **(**Müller and Schwartz, 2006; Müller, 2008).

1.6.4. Progressive brain changes

Structural abnormalities have been observed in SCZ and to a lesser extent in BPD, including decreases in temporal and frontal cortical grey matter volume, and increased ventricular volume (Shenton et al. 2001; Farrow et al. 2005; Moorhead et al. 2007). These abnormalities appear to be progressive, increasing in severity with increased duration of illness (DeLisi et al. 1997; Pantelis et al. 2005). However they are also evident to some extent in prodromal cases (Pantelis et al. 2007), drawing into question whether volume loss precedes illness, causes it, and/or is caused by it. The nature of tissue loss in psychotic disorders is also unclear. The rate of tissue loss is non-linear (DeLisi et al. 1998**)** and lack of gliosis indicates a lack of neurodegenerative processes (Roberts et al. 1986; 1987).

Excessive neural and synaptic pruning during and following adolescence may account for the changes observed (Van Haren et al. 2008), but this theory remains controversial. It is also possible that antipsychotics are responsible for the observed reductions in grey matter (Lieberman et al. 2005; Smieskova et al. 2009; Ho et al. 2011). In BPD, however, lithium treatment appears to increase grey matter volume in rats (Moore et al. 2000) and humans (Sassi et al. 2002), so treatment is a less viable explanation for brain volume loss in BPD.

1.7. Astrocytes

Astrocytes are the most prevalent cell type in the human brain. They are structurally diverse and serve a large range of functions. In grey matter, astrocytes are largely stellate in shape, with a relatively small cell body from which many highly branched processes emerge. The collective branches of these protoplasmic astrocytes completely occupy all grey matter regions, each cell maintaining its own discrete and non-overlapping field of influence. In white matter, most astrocytes take on a less heavily branched morphology with a larger cell body of a more fibrous or striated shape. These fibrous astrocytes lie along and within fiber bundles, where they also maintain continuous and non-overlapping fields of influence (Privat and Rataboul, 1987).

While it is simpler to segregate astrocytes into fibrous and protoplasmic populations, recent research has uncovered robust diversity in the morphology and protein expression of astrocytes throughout the brain, with each cell seemingly honed to the needs of the immediate environment (Kimelberg, 2009**;** Zhang and Barres, 2010; Matyash and

Kettenmann, 2010). This diversity reflects the diverse roles astrocytes serve throughout the brain.

Astrocytes perform a myriad of functions within the central nervous system (CNS). They mediate synaptic development and plasticity, provide structural and metabolic support to surrounding neurons, respond to and release a large range of neurotransmitters, regulate blood brain barrier permeability, and play an active role in the innate inflammatory response and recovery, particularly in repair and scarring processes within the CNS following infection or injury, among many other things. These topics have been reviewed elsewhere (Ullian et al. 2004; Abbott et al. 2006; Pellerin et al. 2007; Buffo et al. 2010; Santello et al. 2012). For the purposes of this thesis, I will focus on roles of astrocytes in developmental, neurotransmission, and inflammatory and CNS repair processes.

1.7.1. Astrocytes and development

In early stages of development, radial glia guide migration of pyramidal neurons from the subventricular zones up into the developing cortex. Radial glia then give rise to immature astrocytes, which migrate through the cortex and begin developing stationary fields of influence at the same time as synapses are beginning to form and develop (Gotz and Barde, 2005). Astrocytes are vital for normal synaptic development, and are deeply involved in synaptic formation, maturation, and pruning. Developing astrocytes guide and encourage synaptic development and activity through the release of soluble factors such as BDNF, thrombospondins, tumor necrosis factor alpha (TNFα), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) (Ullian et al, 2001; Beattie et al.

2002; Barres, 2008**;** Eroglu and Barres, 2010). After synapse formation, many synapses express mostly NMDA receptors, which require preexisting depolarization to open. Astrocytes aid in the activation of these synapses with targeted release of neurotrophins and d-serine, and thus encourage AMPA receptor expression (Eroglu and Barres, 2010). Astrocytes are also deeply involved in the pruning of inactive or silent synapses during critical periods of early and adolescent development, targeting these synapses for degradation (Stevens et al. 2007; Stevens, 2008).

1.7.2. Astrocytes and neurotransmission

Astrocytes perform a myriad of roles in neurotransmission, both directly and indirectly. They are particularly known for their important involvement in glutamate reuptake and metabolism in the glutamate-glutamine cycle, which plays a large role in GABA metabolism as well (Hertz et al. 1999**;** Mathews and Diamond, 2003; Bak et al. 2006). Briefly, Glutamate is removed from the synaptic cleft by glutamate transporters such as excitatory amino acid transporters 1 and 2 (EAAT1; EAAT2), expressed at the astrocyte end feet that surround each synapse. Within the astrocyte, glutamate is either stored for later release as a gliotransmitter, or converted to glutamine by the enzyme glutamine synthetase, and transported out of the astrocyte for surrounding neurons to take in and use to make more glutamate or GABA. Astrocytes also express GABA, and glutamine transporters (Anderson and Swanson, 2000), as well as those for serotonin, norepinephrine, and acetylcholine, among others (Inazu et al. 2001; 2002a; 2002b; 2005). Astrocytes either release these adopted neurotransmitters as their own, or break them down if they are not needed.

Astrocytes also regulate glutamatergic transmission via direct modulation on NMDA receptors. D-serine, an astrocyte-specific gliotransmitter, activates the glycine site of the NMDA receptor, allowing the channel to open. Thus, astrocytes exert permissive control over long-term potentiation and associated memory formation (Panatier et al. 2006; Henneberger et al. 2010**)**. Astrocytes and release a host of other gliotransmitters as well, including nitrous oxide (NO) and arachidonic acid, which contribute to long-term depression and synaptic scaling, among other things (Achour and Pascual, 2010). Furthermore, astrocytes express a large host of neurotransmitter receptors, allowing them to respond dynamically to local events (Auld and Robitaille, 2003; Achour and Pascual, 2010).

1.7.3. Astrocytes, inflammation and CNS repair

Insults to the CNS, including injury, infection, and neurodegeneration all lead to astrocyte activation (Norton et al. 1992). In the event of a trauma or degeneration, astrocytes become able to replicate and migrate to the site of the lesion. Here, astrocytes ramify, or become anisomorphically activated, becoming larger with fewer delicate distal processes (Eng and Ghirnikar, 1994). Ramified astrocytes also express an altered profile of proteins, including increased glial fibrillary acidic protein (GFAP) and vimentin levels (Ridet et al. 1997), and are more structurally rigid. These cells form a permanent glial scar that prevents further damage to surrounding tissue through containment. However, by containing damaged tissue, ramified astrocytes also prevent regrowth and healing in this area (Rudge and Silver, 1990).

Astrocytes also activate in response to immune challenge. The CNS innate immune system is primarily governed by glial cells. While microglia are the most active immune cell type, astrocytes also instrumental in the innate immune response. In the event of an immune insult astrocytes become isomorphically activated, aiding in the recruitment of macrophages and local microglia through interactions with blood brain barrier permeability, and releasing inflammatory cytokine (Ridet et al. 1997; Chen and Swansen, 2003**)**. Astrocytes may also exhibit phagocytic properties, consuming dead and damaged cells and cell fragments following trauma *in vivo* (al-Ali and al-Hussain, 1996; Loov et al. 2012). The full extent of astrocytic immune involvement remains unclear and continues to be a topic of investigation and debate (see Buffo et al. 2010, for discussion).

1.8. Astrocyte-specific proteins

Many proteins exist that are expressed exclusively in astrocytes, but few are expressed at uniform levels across this diverse cell type. Each protein serves distinct roles in day-to-day function, and expression of these proteins can elucidate the activities of the cells they are expressed in (Zhang and Barres, 2010). For the purposes of this dissertation, I will discuss the known roles and expression profiles of four astrocyte-specific proteins: aldehyde dehydrogenase type 1L1 (ALDH1L1), EAAT1, GFAP and vimentin (Figure 1).

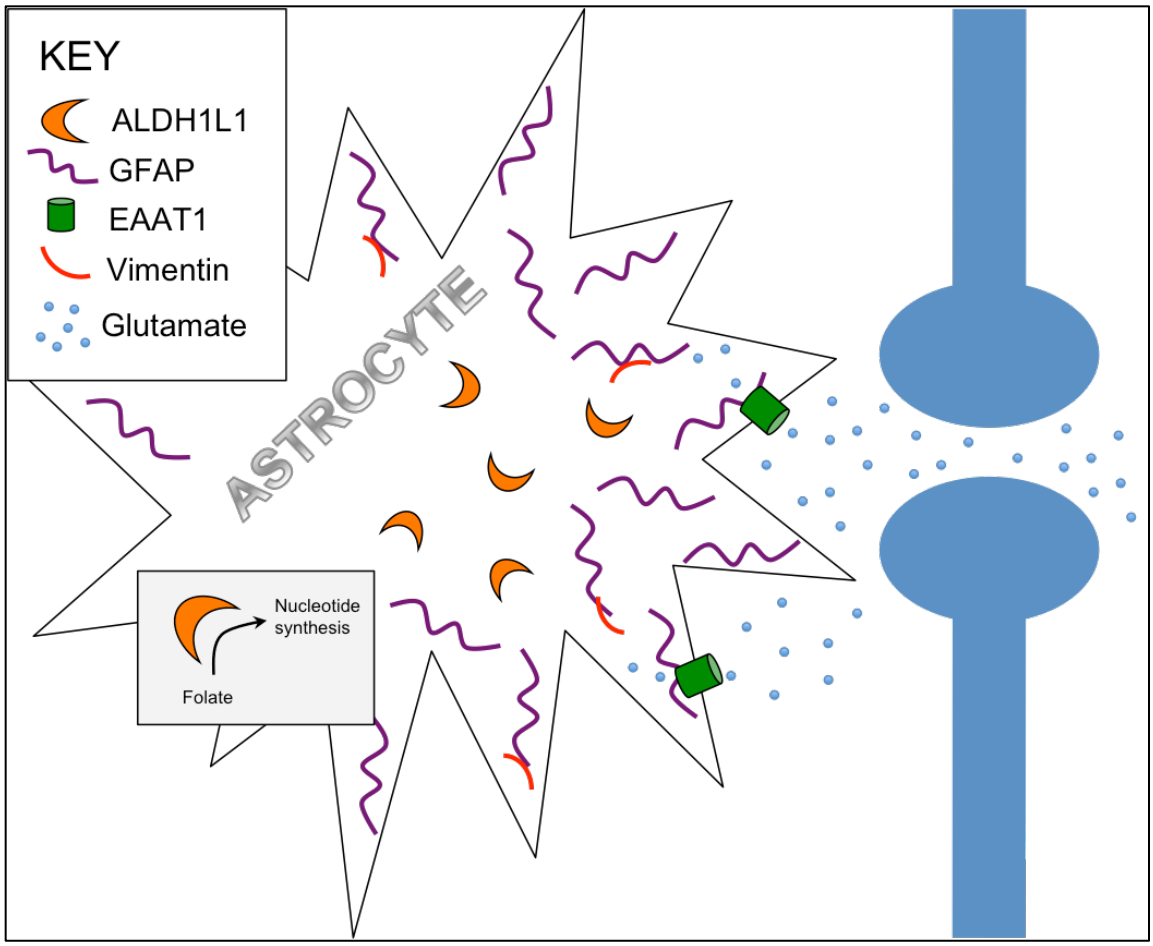

Figure 1: Cell localization and interactions of astrocyte specific proteins ALDH1L1, EAAT, GFAP, and vimentin. ALDH1L1 is located in the astrocyte cytoplasm and is involved with folate metabolism and nucleotide synthesis. EAAT1 in a glutamate transporter that controls extrasynaptic glutamate levels. EAAT1 interacts with GFAP, a scaffolding and structural protein that aids in cellular mobility. Vimentin is another structural protein, primarily expressed in activated astrocytes. ALDH1L1: aldehyde dehydrogenase 1L1; EAAT1: excitatory amino acid transporter 1; GFAP: glial fibrillary acidic protein.

1.8.1. ALDH1L1

ALDH1L1 is a novel astrocyte marker, first suggested as such by Cahoy and colleagues (2008). It is a metabolic protein involved in the processing of aldehydes and more widely expressed in protoplasmic astrocytes than GFAP (Cahoy et al. 2008; Yang

et al. 2011; Olah et al. 2012; see Sofroniew and Vinters, 2010). Before it was identified as an astrocytic marker, ALDH1L1, also known as 10-formyltetrahydrofolate dehydrogenase (FDH), was studied for it's role in folate metabolism and regulation in the liver and kidney (Krupenko, 2009), and its pro-apoptotic effects on tumors throughout the body (Krupenko and Oleinik, 2002; Oleinik et al. 2005). While it is slowly gaining popularity as an astrocyte marker, ALDH1L1 has yet to be used explicitly as a marker for astrocyte populations in the context of psychotic or mood disorders.

1.8.2. EAAT1

EAAT1 is one of two astrocyte-specific glutamate transporters, the other being EAAT2. The functional differences between these two transporters remain unclear (Lee and Pow, 2010). The EAATs are the initiating phase of the astrocyte-driven glutamate-glutamine cycle, taking up glutamate that strays beyond the synapse into the cell for conversion to glutamine (Danbolt, 2001). EAAT1 protein transcription is dynamically regulated in response to extra-synaptic glutamate levels, so changes in EAAT1 protein concentration may indicate changes in extra-synaptic glutamate levels (Gegelashvili and Schousboe, 1997), which can be caused by trauma or a dysregulation of negative feedback regulating glutamatergic output, among other things.

1.8.3. GFAP

GFAP is a structural protein present in all astrocytes to varying degrees, and contributing to structural integrity and flexibility. On astrocyte activation, GFAP is upregulated and its relatively short fiber length facilitates cell motility and migration.

Fibrous astrocytes, found in white matter, express relatively consistent high levels of GFAP. In contrast, constitutively expressed GFAP levels vary dramatically among protoplasmic astrocytes in grey matter, depending on factors such as activation state and proximity to blood vessels (Walz, 2000; Middeldorp and Hol, 2011). Other factors that could affect GFAP levels include changes in vascular density and oxidative stress challenges.

1.8.4. Vimentin

Vimentin is a structural protein expressed primarily in activated astrocytes, and also in immature astrocytes. It is related to GFAP, but its fibers are even shorter and facilitate greater flexibility and movement (Pekny, 2001). Variability in vimentin protein levels would most likely indicate changes in astrocyte activation.

1.9. GFAP and psychotic disease

Astrocyte abnormalities have been reported in SCZ and BPD, however large volumes of conflicting results make this a controversial field of study. GFAP has been shown to be specific and reasonably universal to astrocytes, and thus it is the marker most commonly used to measure astrocytic cell density in grey and white matter in psychiatric disorders (Sonfroniew and Vinters, 2010). Studies of GFAP expression in white matter have been more consistent than those in grey matter, likely because GFAP expression is less variable from cell to cell in white matter. Decreases in GFAP mRNA levels have been reported in the anterior cingulate cortex (ACC) white matter in SCZ and BPD (Webster et al. 2005). These reports match observations made with

immunohistochemistry of decreased GFAP-immunoreactive cells in white matter in this region in SCZ (Williams et al. 2013). However, Katsel and colleagues (2011) failed to observe variation in GFAP mRNA levels in ACC white matter in SCZ. Other authors investigating GFAP protein expression in internal capsule **(**Beasley et al. 2009), and GFAP staining in premotor white matter (Falkai et al. 1999) in SCZ have also observed no change, suggesting that GFAP decreases in white matter may be region-specific.

We chose to focus on grey matter protein expression, where the direction of the results is far less clear. Studies of GFAP protein and/or mRNA expression in cortical grey matter have examined a variety of regions. Directionality of GFAP changes may be regionally specific, with authors reporting decreased GFAP in the ACC in both SCZ and BPD (Steffek et al. 2008), and increases in the DLPFC in SCZ, but not BPD (Toro et al. 2006; Pennington et al. 2008a**;** Martins-de-Souza et al. 2009), however other authors have failed to find changes in GFAP levels in DLPFC (Steffek et al. 2008) and ACC (Katsel et al. 2011) in SCZ. Age may also be a factor in these null results, as both Katsel et al. (2011) and Steffek et al. (2008) used cohorts with average ages in the mid-to-late 70s. Other observations include decreases in four unique isoforms of GFAP protein in SCZ, BPD, and major depressive disorder (MDD), as identified by 2-D electrophoresis in the anterior prefrontal cortex (BA10; Johnston-Wilson et al. 2000**)** and increased GFAP protein levels in the insular cortex in SCZ (Pennington et al. 2008b). A combined analysis of PFC protein variations in SCZ, BPD, and MDD tissue from the Stanley Foundation Neuropathology Consortium observed an overall decrease in GFAP protein and mRNA levels for all psychiatric cohorts in the PFC (BA 8, 9, 10, or 46; Knable et al. 2001).

Immunocytochemical investigations using GFAP as a marker of astrocytes in grey matter have also yielded inconsistent results. Damazdic and colleagues (2001) observed no changes in GFAP immunostaining in SCZ, BPD, or MDD in the entorhinal cortex, while decreases have been reported in GFAP immunostaining in the ACC (Webster et al. 2005; Williams et al. 2013), in keeping with the pattern discussed above in white matter. Rajkowska and colleagues (2002) observed nuanced morphological changes to GFAP-immunoresponsive astrocytes in layer V of the DLPFC in SCZ. There, GFAP area fraction was decreased by 32% while density of positive cell bodies was increased by 81%. The authors suggest that this staining pattern may result from a build-up of GFAP in cell bodies paired with a retraction of processes. This is a sharp example of how GFAP levels may or may not be indicative of astrocyte population counts, and draws into question exactly what proteomic and mRNA studies of GFAP can tell us about the state of astrocytes in pathology.

1.10. Hypotheses and objectives

1.10.1. Hypotheses

We proposed that astrocyte dysfunction is present in DLPFC grey matter in SCZ and BPD. Based on previous literature, we predicted that protein levels of ALDH1L1 would be unchanged, as would vimentin levels (due to lack of gliosis), but that levels of GFAP and EAAT1 (GLAST [glutamate aspartate transporter] in rodents) would be increased, indicating sub-gliotic levels of astrocyte activation, and reflecting altered glutamatergic activity in accordance with the neurotransmitter hypothesis of schizophrenia, discussed above. We expected these changes to be more pronounced in SCZ than in BPD, but that

changes would still be evident in BPD, reflecting the overlapping symptomology and genetics seen in these two disorders. We also predicted that exposure to antipsychotic medication will not affect expression of these proteins in rats. Furthermore, we wished to demonstrate that ALDH1L1 is a more global astrocyte marker than GFAP, labeling both protoplasmic and fibrous astrocytes in human tissue.

1.10.2. Objectives

This project had three objectives: First, to investigate the specific nature of astrocyte dysregulation in psychotic disorder by examining expression of a panel of astrocyte-specific marker proteins. Second, to assess the effects of antipsychotic exposure on astrocyte-specific protein expression in rats. Third, given the limited previous use of ALH1L1, to assess the validity of using ALDH1L1 as an astrocyte marker in human grey matter.

1: *Astrocyte dysregulation in psychotic disorder*: Expression levels of a battery of astrocyte-specific proteins with distinct functions were quantified in order to examine astrocyte dysfunction in SCZ and BPD cohorts. Comparing levels of the astrocyte-specific proteins GFAP, vimentin, ALDH1L1, and EAAT1, in DLPFC of subjects with SCZ, BPD and non-psychiatric controls may help to clarify the specific nature of astrocyte dysregulation in psychotic disorders, differentiating between activation levels, population numbers, and functional abnormalities.

2: *Effects of antipsychotic exposure:* Given that the majority of individuals with psychotic symptoms in our study cohort were prescribed antipsychotic drugs, we examined the effects of typical and atypical antipsychotic medications on astrocyte-associated protein levels in rats.

3. *ALDH1L1 as an astrocyte marker:* ALDH1L1 immunoreactivity was examined in human tissue sections and compared to that of GFAP.

2. Materials and methods

2.1. Human samples

Samples of dorsolateral prefrontal cortex (DLPFC; Brodmann's area 9 (BA9)) were obtained from the Stanley Medical Research Institute (Bethesda, MD, USA). The cohort consisted of 104 subjects: 35 SCZ, 34 BPD and 35 non-psychiatric controls (Table 1). Brain tissue was collected as previously described (Torrey et al. 2000). Briefly, after consent was obtained from next of kin, brains were extracted, hemisected, and frozen in isopentane and dry ice, then areas of interest were dissected and stored at -70C until needed. Diagnoses were made according to Diagnostic and Statistical Manual of Mental Disorders (DSM) IV criteria. Routine neuropathological examinations found no evidence for neurodegenerative changes or pathological lesions. Details of alcohol and illicit drug use, as well as lifetime antipsychotic dose, were available for most but not all subjects. Alcohol and illicit drug use levels were categorized as: low, (none or social use), medium, (moderate past or present use) or high, (heavy past or present use).

Table 1: Subject characteristics.

	Control (n=35)	Bipolar (n=34)	Schizophrenia (n=35)
Age (years)	44.2 ± 7.9	45.4 ± 10.7	42.6 ± 8.5
Sex (M/F)	26/9	16/18	26/9
PMI (hours)	29.4 ± 12.9	37.9 ± 18.6	31.4 ± 15.5
Tissue pH	6.61 ± 0.27	6.43 ± 0.30	6.47 ± 0.24
Alcohol use (Low/Med/High)	30/3/2	12/9/12	16/5/12
Drug use (Low/Med/High)	34/1/0	15/9/9	18/6/9
Psychosis (y/n)	0/35	21/13	35/0
Cause of Death	Cardiac (33) Cancer (1) Asthma (1)	Suicide (15) Cardiac (8) Over dose (5) Drowning (3) Pneumonia (1) Sleep Apnea (1) Ketoacidosis (1)	Cardiac (16) Suicide (7) Pneumonia (5) Over dose (3) Acute Pancreatitis (1) Cirrhosis (1) Exhaustive mania-NMS (1) Motor vehicle accident (1)

	Non-Psychotic (n=46)	Psychotic (n-56)
Age (years)	44.3 +/- 9.27	43.82 +/- 8.94
Sex (M/F)	33/13	34/22
PMI (hours)	32.1 +/- 16.4	33.71 +/- 16.1
Tissue pH	6.60 +/- 0.27	6.42 +/- 0.27
Alcohol use (Low/Med/High)	33/6/7	24/11/18
Drug use (Low/Med/High)	38/4/4	29/11/13
Cause of Death	Suicide (7) Cardiac (35) Over dose (1) Cancer (1) Asthma (1) Sleep Apnea (1)	Suicide (14) Cardiac (21) Over dose (7) Pneumonia (6) Drowning (2) Cirrhosis (1) Sleep Apnea (1) Ketoacidosis (1) Acute Pancreatitis (1) Exhaustive mania-NMS (1) Motor vehicle accident (1)

Data are means ± standard deviation. Abbreviations: PMI, postmortem interval; NMS, neuroleptic malignant syndrome.

2.2. Rat samples

Rats were handled and processed by members of the Beasley and Honer labs, as described in a previous publication (Barakauskas et al 2010). Briefly, adult male Sprague-Dawley rats (Charles River, Montreal, Canada) were divided into three groups balanced for starting weight (270–320g range; n=10 per group). Rats were administered daily intraperitoneal injections of haloperidol (1mg/kg), clozapine (20mg/kg), or vehicle (saline) for 28 days. Rats were then decapitated and cingulate cortex was dissected (Figure 2), flash frozen, and stored at -80°C. All procedures were approved by the University of British Columbia Animal Care Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines.

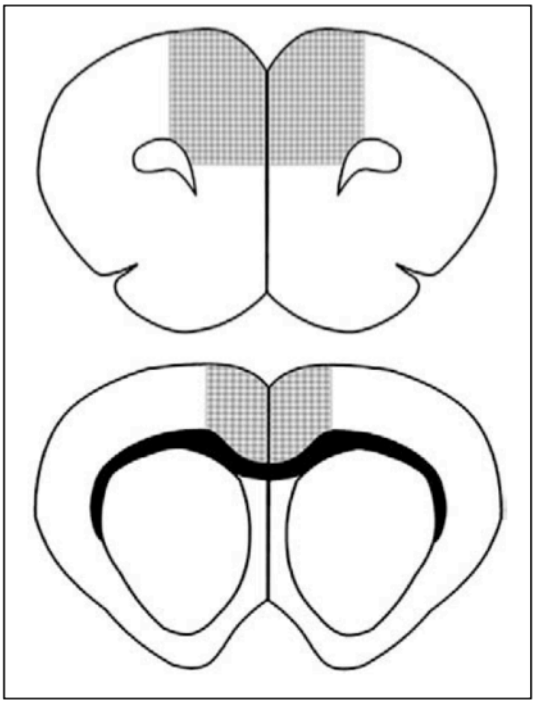

Figure 2: Rat cingulate cortical tissue dissection. Grey matter from the frontal pole to Bregma 0.48 was extracted for analysis (Barakauskas et al, 2010).

2.3. Western blotting

Tissue was homogenized in ten volumes of ice-cold tris-buffered saline (TBS; 0.1M Tris base, 1.4M NaCl, pH7.4) and stored at -80°C. Total protein concentrations were determined using DC Protein Assay kit (BioRad, Hercules, CA, USA). Samples were diluted with Laemmli Sample Buffer (BioRad, Hercules, CA, USA) to 10ug protein/lane, within the linear range of detection for each protein as determined with serial dilutions. Samples were run on duplicate 7.5% or 10% polyacrylamide gels, then transblotted to polyvinylidene fluoride (PVDF) membranes (BioRad, Hercules, CA, USA). Blots were stained with Memcode Reversible Protein Stain (Pierce Biotechnology, Rockford, IL, USA), dried, and imaged with a LAS-3000 Image Analyzer (FujiFilm, Tokyo, Japan) (Figure3). Following imaging, blots were rehydrated, blocked in blocking buffer (5% skim milk powder, 0.05% tween, in TBS), and then incubated overnight at 4°C with primary antibody at experimentally determined concentrations (Table 2). Blots were then washed with blocking buffer 3X10 minutes, and exposed to the appropriate secondary antibody at experimentally determined concentrations (Table 2) for 1 hour at room temperature. Following 2X20 minute washes in TBS with 0.05% tween, and 2X10 minute washes in TBS. Western Lighting chemiluminescence substrate (Perkin-Elmer, Waltham, MA, USA) was applied and blots were imaged with a LAS-3000 Image Analyzer (Fujifilm, Tokyo, Japan).

Blot images were quantified using Science Lab Image Gauge, (FujiFilm, Tokyo, Japan). Three lanes of standard sample, prepared by pooling homogenate from all individual samples, were included on each gel to act as reference. The intensity of each band of interest was normalized both to that of the total protein stain and to the average

band intensity of the adjacent pooled standards. Values of duplicate blots were then averaged (see Table 3 for correlations between duplicate blots**).**

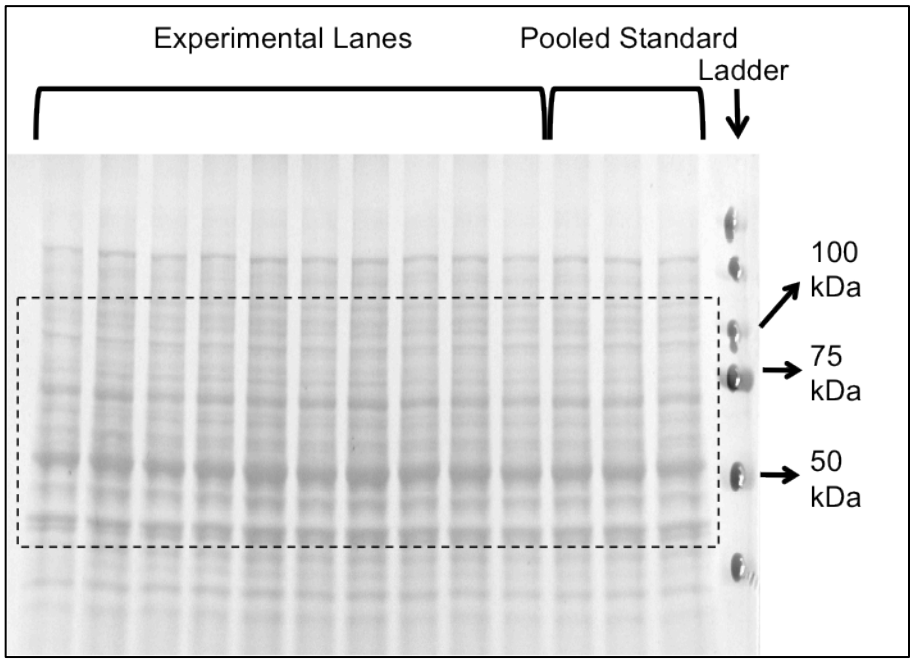

Figure 3: Memcode standardization procedure. Image of an experimental blot stained with Memcode protein stain as a loading control. Protein staining within range of all target proteins was analyzed for each lane (boxed region; 150kDa-37kDa). The first three lanes of each blot contained pooled standard. Protein content of each experimental lane was standardized to the average protein content of the pooled standards from the same blot. After Memcode staining was imaged, blots were immunostained with protein-specific antibody, and immunoresponsive band intensities from each lane were normalized to the standardized protein content of that lane.

Table 2: Antibodies and concentrations used in western blotting.

Target	Product	Source	Concentration
ALDH1L1	Mouse Monoclonal IgG1	Abcam: ab56777	1:500
EAAT1/GLAST	Rabbit Polyclonal (H-50) IgG	Santa Cruz: sc-15316	1:500
GFAP	Rabbit Polyclonal (H-50) IgG	Santa Cruz: sc-9065	1:500
GFAP	Mouse Monoclonal IgG2b	Sternberger Monoclonals: SMI22	1:80,000
Vimentin	Mouse Monoclonal IhG3	Santa Cruz: sc-6260	1:300
Mouse IgG+M	Goat – Peroxidase conjugated	Jackson: 115-035-068	1:2000
Rabbit IgG	Goat – Peroxidase conjugated	Jackson: 111-035-045	1:4000

Table 3: Correlations between duplicated western blots.

		Human	Rat
ALDH1L1	99kDa	r=0.73	r=0.67
	55kDa	r=0.68	r=0.56
	Sum	r=0.64	r=0.72
EAAT1/ GLAST	70-73kDa	r=0.64	r=0.61
GFAP	50kDa	r=0.86	r=0.76
	45kDa	r=0.84	n/a
	Sum	r=0.86	n/a
Vimentin	57kDa	r=0.95	r=0.94

2.4. Immunohistochemistry

Paraffin embedded human frontal cortical gray matter was obtained from the Stanley Medical Research Institute (Bethesda, MD, USA). All experiments were performed on adjacent sections from a single non-psychiatric control case. Two sections were used for each antibody, with an additional section used as a primary antibody-free control.

Sections were dewaxed in xylene 2X10 minutes, and rehydrated with decreasing concentrations of ethanol. Antigen retrieval was performed by submerging slides into ethylenediaminetetraacetic acid (EDTA; 1mM in dH20, pH 8.0) and heating in a microwave for 15 minutes: 2 minutes at 50% power, 3 minutes at 20% power, 10 minutes at 10% power. Sections were then allowed to cool for a further 20 minutes and then rinsed in TBS. Hydrogen peroxide (1.5%) was applied for 20 minutes, followed by TBS rinse 3X10 minutes. Blocking buffer (5% skim milk powder, 2% normal goat serum, in TBS) was applied directly to slides and incubated in a sealed chamber for one hour. Primary antibody (mouse anti ALDH1L1 at 4ug/ml, or mouse anti GFAP at 1:8000) diluted in blocking buffer was then applied and incubated overnight. biotinylated secondary antibody (goat anti-mouse at 1:500; Jackson Immunoresearch, West Grove, PA, USA) was applied and incubated for one hour, followed by application of Vectastain avidin-biotin complex (ABC) reagent (Vector Laboratories, Burlingame, CA, USA) for 20 minutes. Slides were rinsed with TBS 3X10 minutes, and then treated with 3,3'-diaminobenzidine (DAB) kit (Vector Laboratories, Burlingame, CA, USA) according to instructions. Sections were then rinsed in deionized water for 5 minutes, dehydrated with increasing concentrations of ethanol, and exposed to xylene 2X5 minutes. Slides were

coverslipped with Permount mounting media (Fisher Scientific, Ottawa, Ontario, Canada), and stored in dark boxes until analysis.

Slides were analyzed and imaged with an Olympus BX61 light microscope with Olympus DP71 camera and Prior ProScan II motorized stage system (Rockland, MA, USA), coupled with the software Image-Pro Plus (version 6.3.1.542; Media Cybernetics, Silver Spring, MD). Images were assessed qualitatively for distribution of staining across grey and white matter, as well as staining distribution within cells. No quantitative analyses were conducted.

2.5. Statistical analysis

Statistical analyses were performed using SPSS 11.0.4 for Mac. Data were transformed where necessary to conform to a normal distribution according to Kolmogorov-Smirnov and Shapiro-Wilk tests. Between-group differences in potential confounders such as age, postmortem interval (PMI), brain pH and sex were examined using Analysis of Variance (ANOVA) or X^2 tests. The relationship between these potential confounders and immunoblot band intensities were examined using Spearman's Rank Correlations or ANOVAs. Analysis of Covariance (ANCOVA) was then used to assess differences in band intensity between diagnostic groups. Where potential confounders significantly correlated with band intensity, or significantly differed between groups, the variable was included as a covariate or secondary fixed factor in that analysis. Contrast analysis between control and each diagnostic group were also performed within the context of ANCOVA analysis.

In addition, we assessed whether astrocyte-specific protein levels were altered in patients who experienced psychosis. Cases from all three diagnostic groups were recategorized into psychotic (n=56) or non-psychotic (n=46) cohorts. The effect of psychosis on band intensities was examined as described above.

The potential effects of drug or alcohol use on protein expression were assessed independently, using ANOVA. Effects of lifetime antipsychotic dose on protein expression were assessed using Spearman's Rank Correlation. Relationships between suicide and protein expression were assessed using ANOVA.

The effects of antipsychotic treatment on band intensity in rodents were assessed using ANOVA. For all tests, alpha=0.05.

3. Results

3.1. Western blotting results in humans

3.1.1. Raw data observations

Astrocytic protein levels were determined through western blotting. Images of typical blots can be seen in Figure 4. Bands of interest were identified based on current literature, and their intensities quantified. When data sets did not conform to a normal distribution according to Kolmogorov-Smirnov tests**,** they were transformed in order to do so. In instances where multiple bands were measured, bands associated with the same protein were consistently highly correlated with each other (r >0.78) and were assessed both separately and combined into sum values.

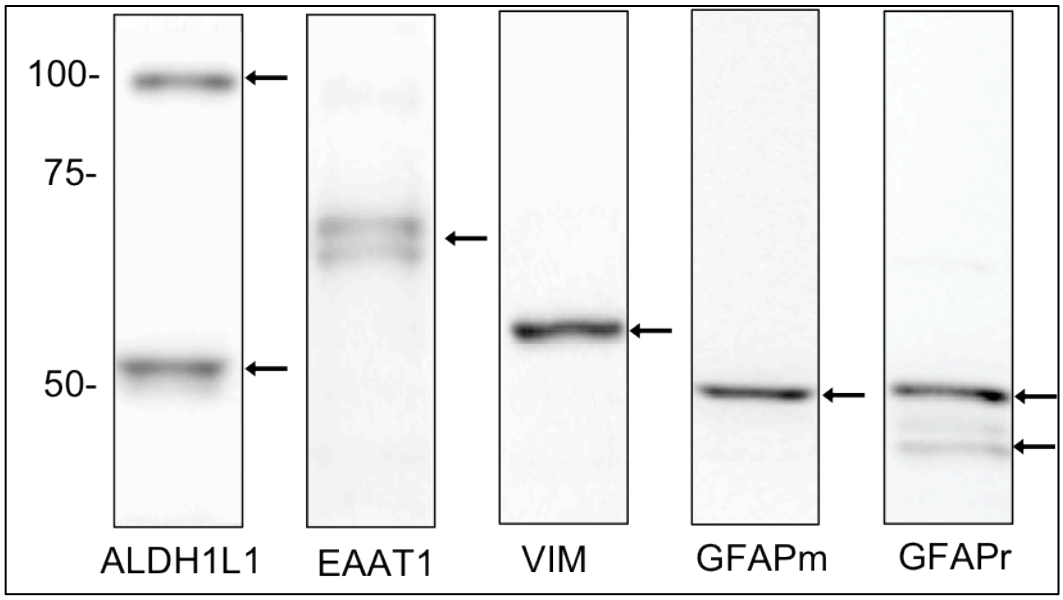

Figure 4: Immunostained blots. Images of immunostained western blots of human DLPFC, showing typical band distributions in response to the antibodies used. All lanes shown are pooled standard samples. Arrows indicate bands used in analysis. **Examples of previous studies using these antibodies are cited below:**
ALDH1L1: mouse-anti-ALDH1L1, Abcam 1:500 (Chen et al. 2012, PMID:21987076). EAAT1: rabbit-anti-EAAT1, clone H-50, Santa Cruz 1:500 (Bauer et al. 2010, PMID:19716271). VIM: mouse-anti-vimentin, V9, Santa Cruz 1:300 (Knabe et al. 2008, PMID:18335540). GFAPr: rabbit-anti-GFAP, Santa Cruz 1:500 (Borkham-Kamphorst et al. 2011, PMID:21457438). GFAPm: mouse-anti-GFAP, SMI-22, Cedarlane 1:8,000 (McLendon et al. 1986, PMID:3534145; Talos et al. 2006, PMID:16680782).

ALDH1L1

Two bands were analyzed on ALDH1L1 blots, at 55kDa and 99kDa. ALDH1L1 has a predicted molecular weight of 99kDa, and it is likely that the observed 55kDa band is a breakdown product. Schirch and colleagues (1994) observed and documented this band, and suggested that it is the result of hydrolase activity of 2-mercaptoethanol exposure. The sum of these two bands was also analyzed to provide a more complete picture of ALDH1L1 expression in these samples. While the 99kDa data sets conformed to a normal distribution and did not require transformation, the natural log of the 55kDa data set, and the square root of the sum of both value sets were used for statistical analysis.

EAAT1

Two bands were identified on EAAT1 blots. These bands, located between 70kDa and 72kDa, were analyzed together due to their close proximity. As described by previous authors, glutamate transporters frequently present in western blotting as a doublet, or a smudge, due to glycosylation heterogeneity of two different isoforms of similar molecular weights (Schulte and Stoffel, 1995). For this data set to conform to a normal distribution, the square roots of the data were analyzed.

GFAP

Two bands were analyzed on GFAP blots, at 45kDa and 50kDa. The predicted molecular weight of GFAP is between 45kDa and 55kDa, representing several different isoforms. GFAP is known to have multiple splice variants and phosphorylation states, each likely to serve related but varied functions in the cell (Johnston-Wilson et al. 2000).

We observed several bands within this range; two major bands at 45 and 55kDa, as well as multiple faint bands. We chose to only analyze the two most prominent bands, as the less prominent bands did not replicate reliably. The sum of these two bands was also analyzed to provide a more complete picture of GFAP expression in these samples. The square roots of the 50kDa and sum GFAP data sets, and the cube root of the 45kDa, were used for statistical analysis.

VIMENTIN

One band was present on vimentin blots at the expected molecular weight of 57kDa. The normal logs of these data were used for statistical analysis.

3.1.2. Differences in demographic variables between groups

Brain pH ($F(2,103)=4.174$, $p=0.018$) and sex ($X^2=7.344$, $p=0.025$) differed significantly between diagnostic groups. Brain pH differed significantly between psychotic and non-psychotic cohorts ($F(1,101)=10.887$, $p=0.001$). No variations in age or PMI were observed between groups or by psychosis (see Table 4).

Table 4: Differences in Age, PMI, pH, and sex, between groups and by psychosis.

	Age	PMI	pH	Sex (Chi^2)
Psychosis (ANOVA)	F=0.065 (p=0.799)	F=0.247 (p=0.620)	F=10.887 **(p=0.001)**	X^2=1.373 (p=0.241)
Diagnosis (ANOVA)	F=0.870 (p=0.422)	F=2.713 (p=0.071)	F=4.174 **(p=0.018)** BPD **p=0.006** SCZ **p=0.043**	X^2=7.344 **(p=0.025)**

Abbreviation: PMI, post mortem interval. Chi square tests performed between sex and group, and sex and psychosis. Significance (p) values are **bolded** where $p<0.05$.

3.1.3. Effect of demographic variables on protein expression

Many factors (e.g. age, PMI, brain pH) may influence protein expression in human tissue. Thus, we tested for relationships between these potentially confounding variables and protein levels (see Table 5).

ALDH1L1

The 55kDa ALDH1L1 band correlated with brain pH ($r=-0.194$, $p=0.049$), while the 99kDa band ($r=0.271$, $p=0.006$), and sum of both bands ($r=0.276$, $p=0.005$), correlated with age. ALDH1L1 protein levels did not correlate with PMI or vary between sexes.

EAAT1

The EAAT1 70-72kDa band intensity correlated with PMI ($r=-0.269$, $p=0.006$). This correlation has been attributed by previous authors to proteolytic cleavage (Li et al. 2012), and will be discussed in more detail below. EAAT1 protein levels did not correlate with age, or pH, nor did they vary between sexes.

GFAP

The expression of both 50kDa and 54kDa GFAP bands individually, as well as their sum, correlated with brain pH (45kDa: $r=-0.419$, $p<0.001$; 50kDa: $r=0.195$, $p=0.048$; sum: $r=-0.269$, $p=0.006$) and PMI (45kDa: $r=-0.421$, $p<0.001$; 50kDa: $r=-0.443$, $p<0.001$; sum: $r=-0.428$, $p<0.001$). GFAP protein levels did not correlate with age, nor did they vary between sexes.

Table 5: Relationship between protein expression and the variables age, PMI, pH and sex.

		Age	PMI	pH	Sex
ALDH1L1	99kDa	r=0.271 **p=0.006**	r=-0.074 p=0.460	r=0.091 p=0.361	p=0.257
	55kDa	r=0.180 p=0.069	r=-0.194 **p=0.049**	r=-0.159 p=0.108	p=0.062*
	Sum	r=0.276 **p=0.005**	r=-0.152 p=0.126	r=-0.048 p=0.631	p=0.145
GFAP	50kDa	r=0.060 p=0.544	r=-0.195 **p=0.048**	r=-0.443 **p<0.001**	p=0.738
	45kDa	r=0.060 p=0.545	r=-0.419 **p<0.001**	r=-0.421 **p<0.001**	p=0.438
	Sum	r=0.018 p=0.853	r=-0.269 **p=0.006**	r=-0.428 **p<0.001**	p=0.663
Vimentin	57kDa	r=-0.051 p=0.608	r=-0.194 **p=0.048**	r=-0.237 **p=0.015**	p=0.274
EAAT1	70kDa	r=-0.032 p=0.750)	r=-0.162 p=0.105	r=-0.269 **p=0.006**	p=0.178

Age, PMI and pH correlations were examined using Spearman's Rank. Effects of sex were analysed using ANOVA. Abbreviation: PMI, post mortem interval. Significance (p) values are **bolded** where $p<0.05$.

VIMENTIN

The 57kDa vimentin band intensity correlated with PMI (r=-0.237, p=0.015) and pH (r=-0.194, p=0.048). Vimentin protein levels did not correlate with age nor did they vary between sexes.

3.1.4. Effect of diagnostic group

Controlling for the confounding variables as necessary, we determined whether protein levels differed between diagnostic groups using ANCOVAs.

ALDH1L1

Brain pH, age (for 99KDa and sum), and sex were included in the analysis. No significant group differences were observed (Figure 5A; 6A).

GFAP

Brain pH, PMI and sex were included in analysis. The sum of GFAP bands differed between diagnostic groups (ANCOVA, $F(2,103)=3.361$, $p=0.039$). Contrast analysis indicated significant increases in sum GFAP band intensity in BPD ($p=0.044$) and SCZ ($p=0.017$) relative to controls. The 45kDa and 50kDa GFAP did not differ significantly between groups (45kDa $F(2,103)=1.909$, $p=0.154$; 50kDa: $F(2,103)=2.903$, $p=0.060$), although contrast analysis indicated that the 50kDa band ($p=0.020$) was significantly increased in SCZ, with a similar trend in the 45kDa band ($p=0.067$) (Figure 5B; 6B).

EAAT1

Brain pH, PMI and sex were included in the analysis. No significant group difference was observed (Figure 5C; 6C).

Vimentin

Brain pH, PMI and sex were included in analysis. No significant group difference was observed (Figure 5D; 6C).

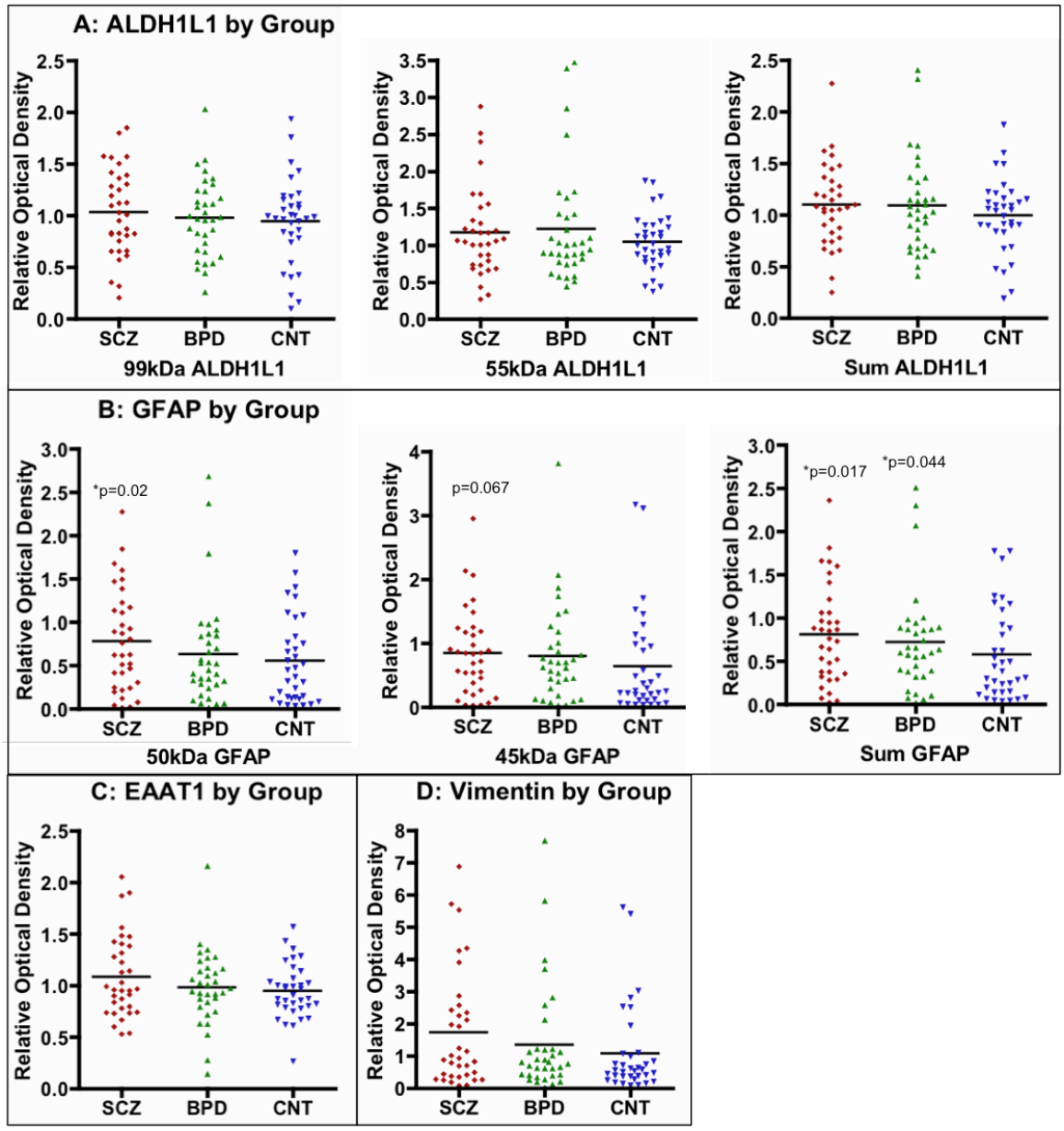

Figure 5: GFAP increased in schizophrenia, no other proteins altered. ALDH1L1 (55kDa, 99kDa, summed bands; A), GFAP (45kDa, 50kDa, summed bands; B), EAAT1 (C), and vimentin (D) protein expression was compared between SCZ (n=35), BPD (n=34) and control (CNT n=35) groups by ANCOVA. Data represented as untransformed values with means represented by a bar. Overall, the sum of the GFAP bands differed between diagnostic groups ($F(2,103)=3.361$, $p=0.039$). Contrast analysis indicated significant increases in sum GFAP band intensity in BPD and SCZ relative to controls. Overall, the 45kDa and 50kDa GFAP bands did not differ significantly between groups although contrasts indicated that the 50kDa band was significantly increased in SCZ, with a similar trend in the 45kDa band. Significance values (contrast analysis, compared against the CNT group) are shown where $p<0.10$. Asterisks (*) indicate $p<0.05$.

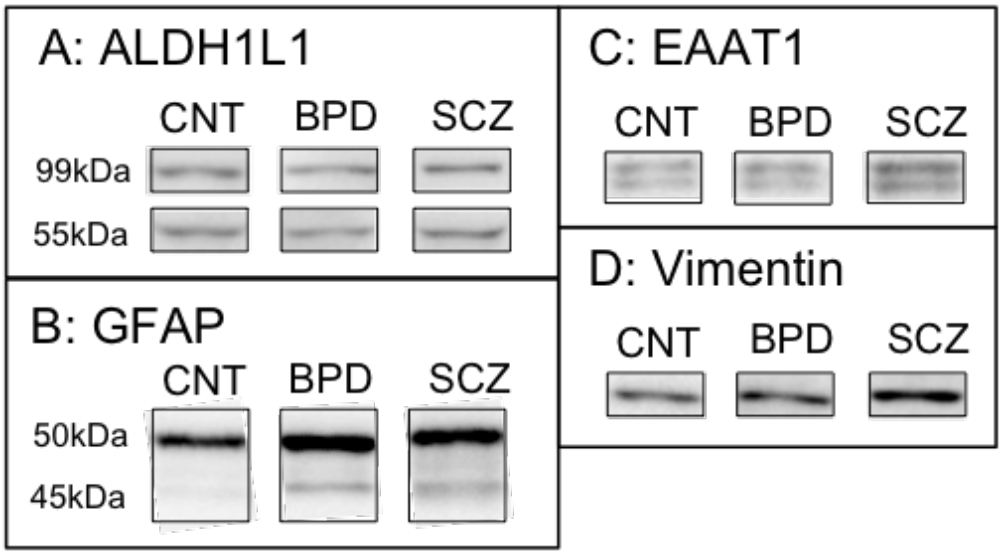

Figure 6: Representative blots: Examples of bands from western blots immunoresponsive to antibodies against ALDH1L1 (A), GFAP (B), EAAT1 (C) and vimentin (D), in individuals with SCZ, BPD, and non-psychiatric controls (CNT).

3.1.5. Effects of psychosis

We next looked at the effect of psychosis on protein expression. We segregated our samples into psychotic vs. non-psychotic cohorts, and compared protein expression in these two groups with ANCOVA. When cases were divided into psychotic and non-psychotic cohorts, the 45kDa band, 50kDa band, and the sum of the GFAP bands were all found to be significantly increased in psychosis (45kDa: $F(1,101)=6.895$, $p=0.010$; 50kDa: $F(1,101)=5.410$, $p=0.022$; sum: $F(1,101)=6.808$, $p=0.010$). No significant differences were observed in ALDH1L1, EAAT1, or vimentin levels (Figure 7).

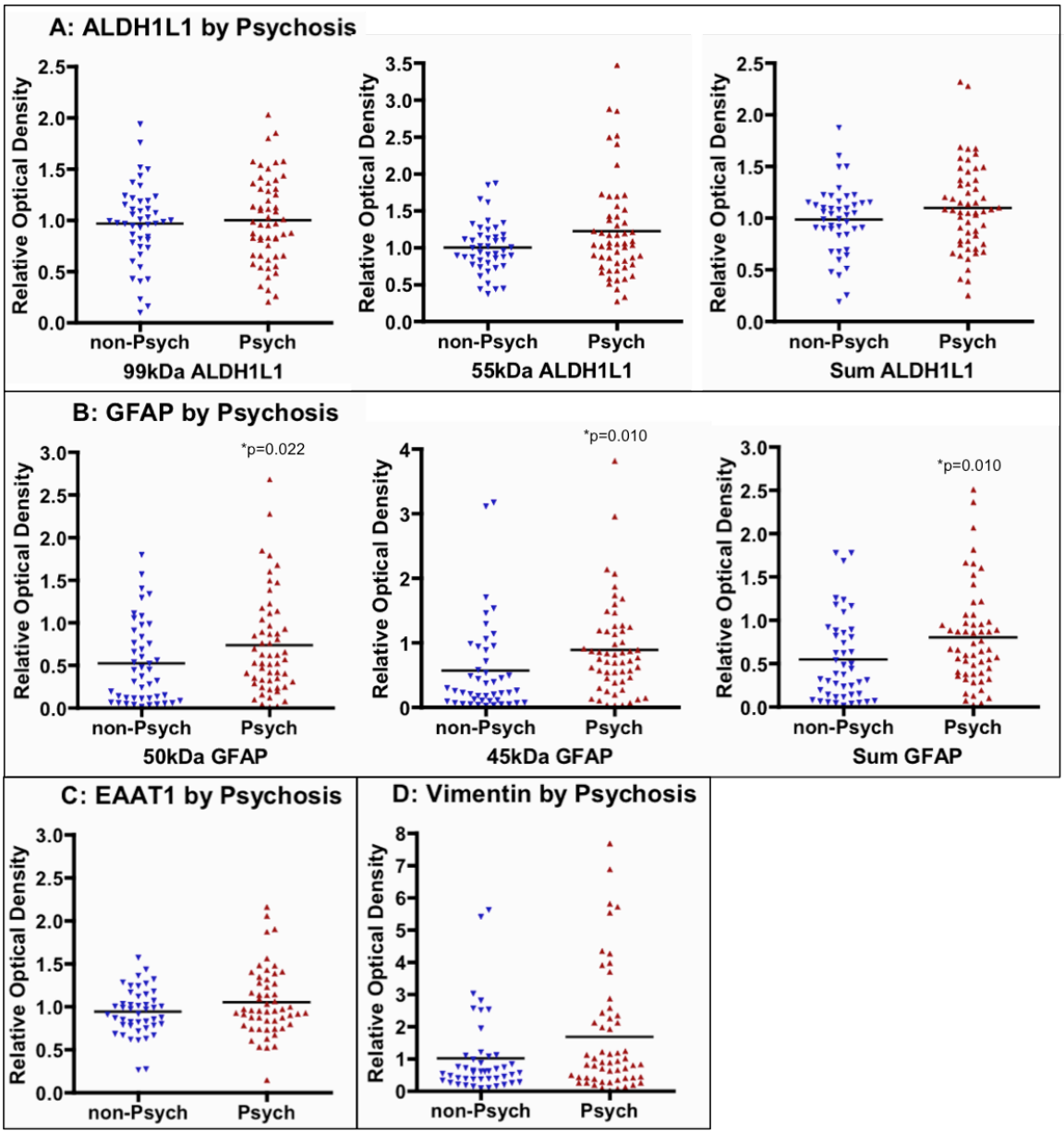

Figure 7: GFAP increased with psychosis, no other proteins affected. ALDH1L1 (55kDa, 99kDa, and summed bands; A), GFAP (45kDa, 50kDa, and summed bands; B), EAAT1 (C), and vimentin (D) protein expression was compared between psychotic (Psych; n=56) and non-psychotic (non-Psych; n=46) groups, using ANCOVA. The 45kDa band, 50kDa band, and the sum of the GFAP bands were all significantly increased in psychosis. Data is represented as untransformed values with means represented by a bar. Significance values are shown where $p<0.10$. Asterisks (*) indicate $p<0.05$.

3.1.6. External factors that may influence protein expression

3.1.6.1. Effects of pH and PMI

Brain pH was significantly different between diagnostic groups, and correlated with the expression of the majority of proteins we assessed. In addition, PMI correlated with GFAP, EAAT1, and vimentin. Previous authors have observed that extreme brain acidity, (low pH; Kingsbury et al. 1995; Li et al. 2004; Stan et al. 2006) and PMI greater than 24 hours (Stan et al. 2006; Ferrer et al. 2008), substantially increased protein and mRNA degradation. This phenomenon has also been observed in tissue prepared by the Stanley Foundation Neuropathology Consortium (Webster, 2006). We thus investigated the effects of removing from our analysis individuals with pH values within the first quartile ($pH < 6.3125$), and individuals with PMI greater than 24 hours. The remaining group distribution is as follows: for pH analysis, CNT=30, BPD=24, SCZ=24, Psych=37, Non-Psych=39; for PMI analysis, CNT=11, BPD=10, SCZ=9, Psych=15, Non-Psych=14.

PH

Within the upper three quartiles of the pH distribution, sum GFAP was significantly increased in BPD when compared directly to controls ($p=0.025$). Trends towards increased GFAP levels were also observed in BPD for both the 50kDa band ($p=0.090$) and the 45kDa band ($p=0.055$). The 45kDa GFAP was significantly increased in individuals with psychosis ($F(1,75)=5.998$, $p=0.017$), and a similar trend was observed towards increased sum GFAP in psychosis ($F(1,75)=3.268$, $p=0.075$).

We also observed significant variation between groups in EAAT1 levels in this subset of individuals, with increased EAAT1 in SCZ ($F(2,77)=9.045$, $p=0.013$). EAAT1 levels did not vary with psychosis. ALDH1L1 and vimentin did not significantly vary between groups or with psychosis.

PMI

Removal of individuals with a PMI greater than 24 hours led to GFAP changes similar to those seen in the complete cohort, with 50kDa and sum GFAP levels significantly differing between diagnostic groups (50kDa: $F(2,29)=7.315$. $p=0.038$; sum: $F(2,29)=6.714$, $p=0.041$). Contrast analysis showed that these changes were primarily due to increases in GFAP expression in SCZ rather than BPD (50kDa: SCZ $p=0.034$, BPD $p=0.927$; Sum: SCZ $p=0.034$, BPD $p=0.707$). There was a trend toward variation in 45kDa GFAP expression between diagnostic groups that did not reach significance ($F(2,29)=3.680$, $p=0.097$). All measurements of GFAP were significantly increased in psychotic individuals when compared to non-psychotic individuals (50kDa: $F(1,28)=7.049$, $p=0.014$; 45kDa: $F(1,28)=5.845$, $p=0.023$; Sum: $F(1,28)=7.758$, $p=0.01$). No other proteins varied between diagnostic groups, however, in this subset of samples EAAT1 protein levels were increased in psychotic individuals ($F(1,28)=4.559$, $p=0.043$).

3.1.6.2. Effect of sex

There were a greater number of females in the BPD group (n=18) than there were in the other groups (n=9 for each), and so we controlled for sex as an external factor in ANCOVA analyses. We failed to find a main effect of sex on astrocyte protein

expression, or any sex by diagnosis interaction. However there is a large body of literature supporting sex-based differences in the development and presentation of both BPD and SCZ (Abel et al. 2010; Diflorio and Jones, 2010). To test the possibility that astrocyte proteins vary with diagnosis or psychosis differently in males and females, we assessed protein expression by group in each sex separately, using ANCOVA analysis. Covariates were reassessed in the context of each sex.

MALES

Our cohort contains a total of 68 males: 26 control individuals, 16 BPD individuals, and 26 SCZ individuals. Of those, 34 experienced psychosis and 33 did not. The psychotic status of one individual was unknown. We found that much of the same covariates applied in males as they did overall, with the exception that PMI correlated with 55kDa ALDH1L1, but did not correlate with EAAT1 or vimentin. In the male cohort no proteins significantly varied between groups or by psychosis using ANCOVA analysis.

FEMALES

Our cohort contains a total of 36 females: 9 control individuals, 18 BPD individuals, and 9 SCZ individuals. Of those, 22 experienced psychosis and 13 did not. The psychotic status of one individual was unknown. In the female cohort, brain pH did not correlate with any protein expressions, nor did it vary with diagnosis or psychosis, and was therefore not used as a covariate. In addition, 99kDa ALDH1L1 did not correlate with

age. All measurements of GFAP still correlated with PMI, as was the case with vimentin, however EAAT1 did not correlate with PMI.

While the covariates differed from what was observed overall, the results from the subsequent ANCOVAs look very similar. When assessed by diagnosis, we see trends towards between-group variability in 50kDa GFAP ($F(34,2)=2.940$, $p=0.067$), with contrast analysis uncovering a trend towards increased 50kDa GFAP in BPD ($p=0.063$) and significantly increased 50kDa GFAP in SCZ ($p=0.028$). A trend towards variability was also seen in overall GFAP ($F(2,34)=2.873$, $p=0.071$), with a significant increase in SCZ ($p=0.033$) and a trent towards increased sum GFAP in BPD ($p=0.055$). In psychosis, this variability is even more pronounced, with significant increases in all measurements of GFAP associated with psychosis (50kDa: $F(1,34)=7.290$, $p=0.011$; 45kDa: $F(1,34)=11.807$, $p=0.002$; Sum:$F(34(1)=9.351$, $p=0.004$).

3.1.6.3. Effect of suicide

We assessed the effects of suicide and found no impact. The majority of suicide cases occurred in non-psychotic BPD individuals.

3.1.6.4. Effect of antipsychotics

We also assessed the relationship between estimated total lifetime antipsychotic medication (in fluphenazine milligram equivalents; Torrey et al. 2000) and astrocytic protein levels. No significant correlations were observed between any protein expression and lifetime antipsychotic dose. However, as we did not know the type of medications prescribed (typical or atypical), or the duration of treatment, it is difficult to assess the

importance of these findings. We are aware that 23 out of 34 BPD patients were prescribed mood stabilizers such as lithium or valproate, but the amount and whether prescribed medications were taken remains unknown.

3.1.6.5. Effect of illicit drug use

The use of illicit substances such as cocaine, heroin, and marijuana, can dramatically effect neurological functioning both acutely and over time, and their use is significantly increased in the psychiatric community (Regier et al. 1990; Ringen et al. 2008).

In our cohort, 19 individuals were heavy drug users, 16 individuals were moderate drug users, and 67 individuals were casual drug users or non-users, however the type of drug being used is unknown. Significant differences in illicit drug usage were observed between diagnostic groups (X^2= 31.758, $p<0.001$) and between psychotic and non-psychotic individuals (X^2= 9.857, $p=0.007$) (See Table 1 for group distributions**)**. We found that the sum GFAP band intensity was altered with illicit drug use ($F(2,103)=3.084$, $p=0.050$), with GFAP being increased in subjects with "high" drug use relative to "low" use ($p=0.019$). Vimentin band intensity was also altered with drug use ($F(2,103)=8.489$, $p<0.001$), being increased in subjects with "high" drug use relative to "low" use ($p<0.001$) (Figure 8).

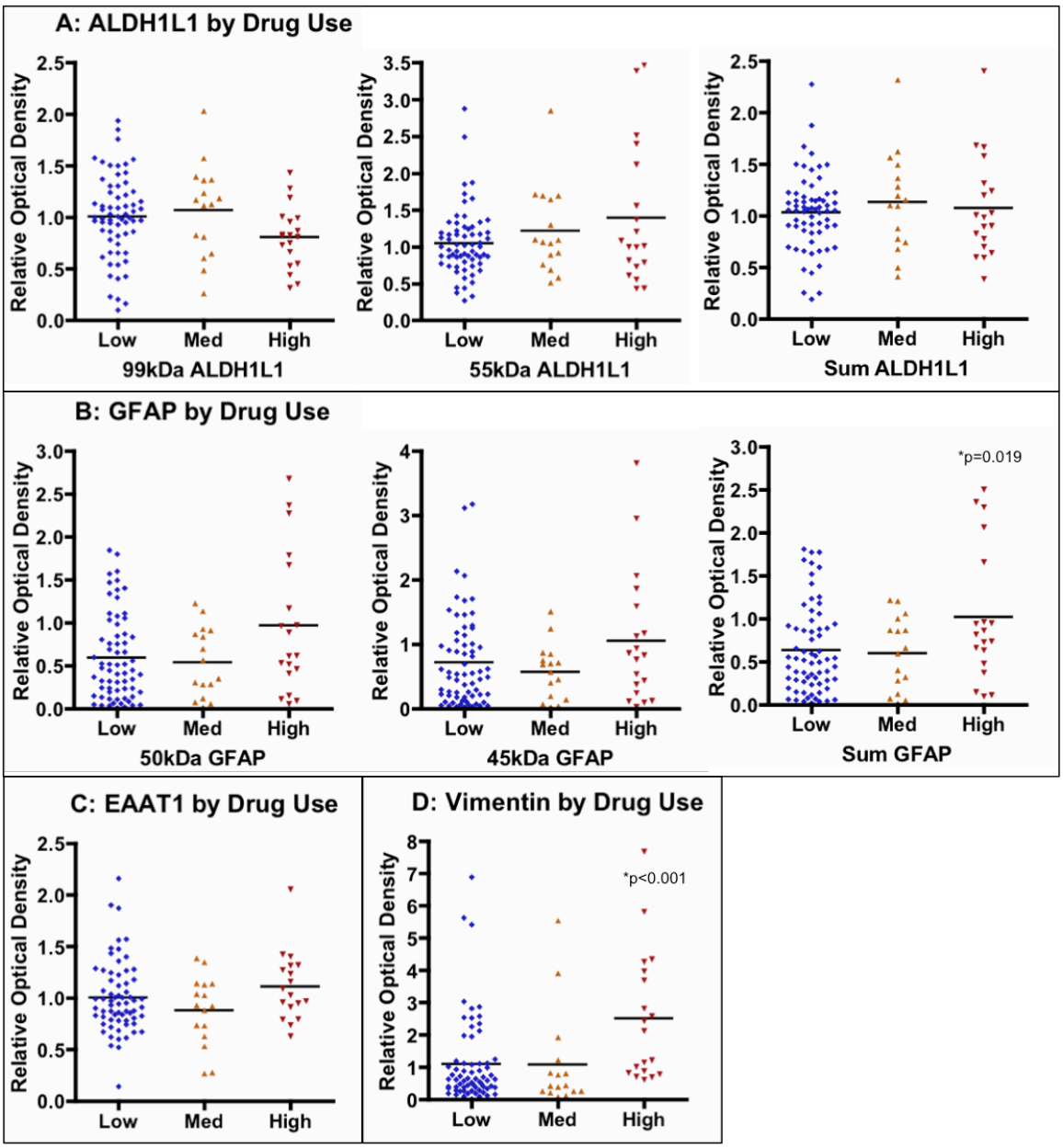

Figure 8: Drug use influences GFAP and vimentin levels. ALDH1L1 (55kDa, 99kDa, and summed bands; A), GFAP (45kDa, 50kDa, and summed bands; B), EAAT1 (C), and vimentin (D) protein expression was compared between individuals with histories of low (Low; n=67), medium (Med; n=16), or high (High; n=19) drug use. Overall, sum GFAP band intensity was altered due to illicit drug use ($F(2,103)=3.084$, $p=0.050$), with GFAP increased in subjects with "high" drug use relative to "low" use. Vimentin band intensity was also altered overall ($F(2,103)=8.489$, $p<0.001$), being increased in subjects with "high" drug use relative to "low" use. Data is represented as untransformed values with means represented by a bar. Significance values (contrast analysis, compared against the "low" group) are shown where $p<0.10$. Asterisks (*) indicate $p<0.05$.

When individuals with "high" illicit drug use were removed from analysis (leaving CNT: n=35, BPD: n=24, SCZ: n=24, Psych: n=42, Non-Psych: n=40), GFAP sum protein levels were still significantly increased in SCZ when compared directly to controls (p=0.049) and also increased in psychosis (F(1,81)=4.112, p=0.046). Vimentin levels failed to significantly differ between diagnostic groups or by psychosis in low and moderate drug users.

3.1.6.6. Effect of alcohol use

Both acute and chronic exposure to alcohol causes a myriad of changes in the brain. Alcohol is heavily used in the psychiatric community, and may affect astrocyte protein expression **(**Margolese et al. 2004).

In our cohort, 26 individuals were heavy alcohol users, 18 individuals were moderate alcohol users, and 59 individuals were casual alcohol users or non-users. Significant differences in alcohol use levels were observed between diagnostic groups (X^2=10.649, p=0.005), as well as psychotic vs. non-psychotic individuals (X^2= 7.352, p=0.025) (See Table 1 for group distributions). Vimentin band intensity was altered with alcohol use (F(2,103)=3.533, p=0.033), being increased in subjects with "high" alcohol use relative to "low" use (p=0.037). No other protein levels interacted with alcohol use (Figure 9).

When individuals with high alcohol use were removed from analysis (leaving CNT: n=33, BPD: n=21, SCZ: n=23), vimentin failed to significantly differ between diagnostic groups or by psychosis, however, a trend towards altered vimentin levels between groups (F(2,75)=5.702, p=0.088) is apparent.

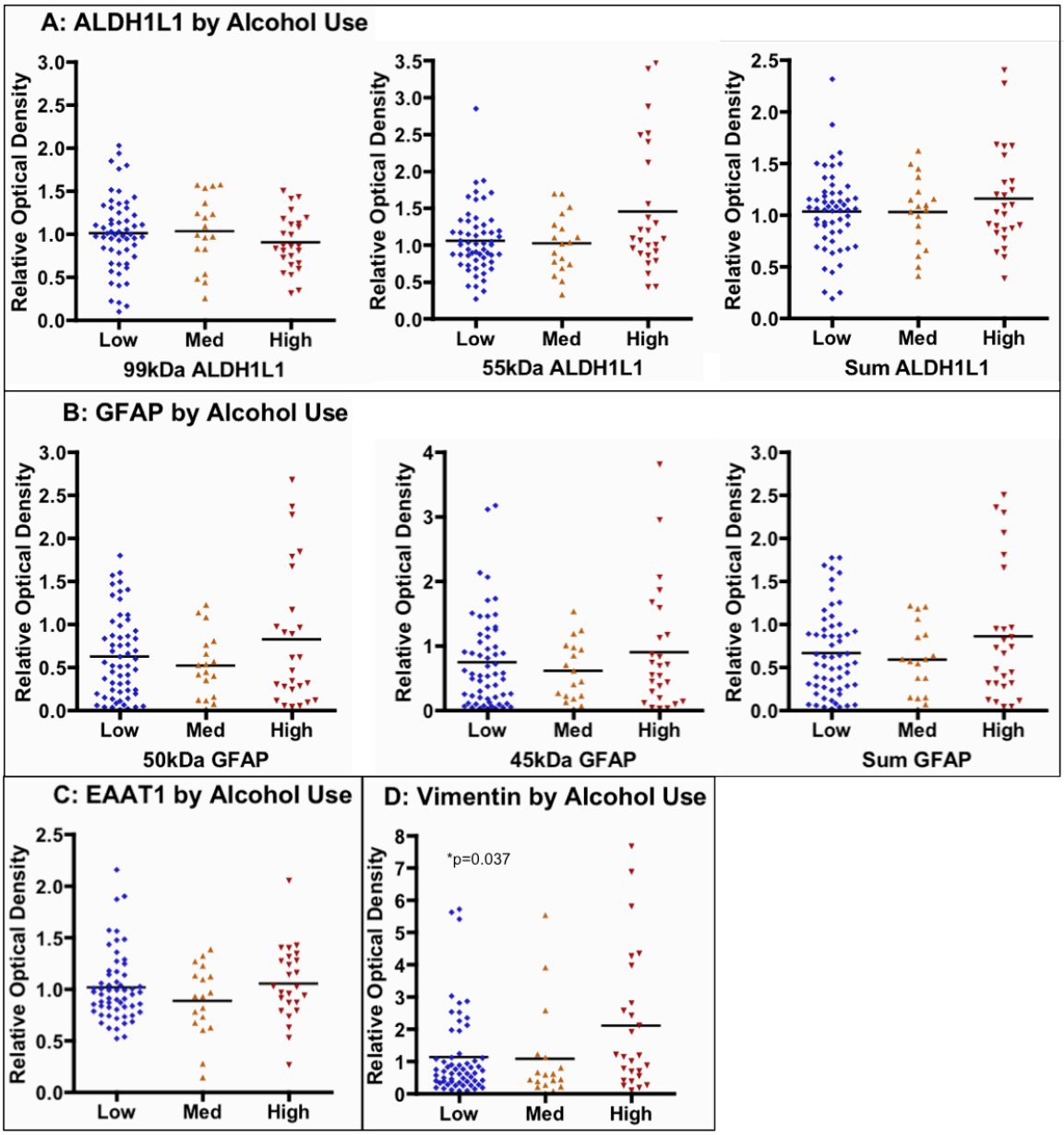

Figure 9: Alcohol use influences vimentin expression. ALDH1L1 (55kDa, 99kDa, and summed bands; A), GFAP (45kDa, 50kDa, and summed bands; B), EAAT1 (C), and vimentin (D) protein expression was compared between individuals with histories of low (Low; n=59), medium (Med; n=18), or high (High; n=26) alcohol use. Vimentin band intensity was altered with alcohol use ($F(2,103)=3.533$, $p=0.033$), being increased in subjects with "high" alcohol use relative to "low" use. Data is represented as untransformed values with means represented by a bar. Significance values (contrast analysis, compared against the "low" group) are shown where $p<0.10$. Asterisks (*) indicate $p<0.05$.

3.2. Western blotting results in rats

3.2.1. Raw data observations

Bands observed in rat tissue were similar to those seen in humans, with the exception of GFAP. Because the polyclonal rabbit-anti-GFAP antibody bound non-specifically to rat tissue, we used a different antibody with the rat samples. The monoclonal mouse-anti-GFAP antibody produced one band at 50kDa (see Figure 5: GFAPm), which required a natural log transformation to adhere to a normal distribution. ALDH1L1 99kDa data set did not require a transformation, while the 55kDa and sum data sets were transformed with natural log. Vimentin was also transformed with natural log, and the square root of the EAAT1 data set was taken so that data conformed to a normal distribution.

3.2.2. Effect of antipsychotics on astrocyte-associated proteins in rats

Rats exposed to antipsychotics did not exhibit significant differences in band intensities for any astrocytic protein overall. However, trends were observed in the 99kDa and sum ALDH1L1 bands (99kDa: $F(2,29)=3.252$, $p=0.054$; sum: $F(2,29)=2.704$, $p=0.085$), with contrast analysis indicating significant increases in 99kDa and sum ALDH1L1 band intensity with clozapine treatment (99kDa: $p=0.019$, sum: $p=0.04$), and trends towards increased expression of sum ALDH1L1 with haloperidol treatment ($p=0.077$), when compared directly to saline treatment. A trend was also observed in GFAP ($F(2,29)=2.683$, $p=0.087$), which carried over in contrast analysis to a non-significant trend towards increased expression in haloperidol ($p=0.065$), but not clozapine. (Figure 10).

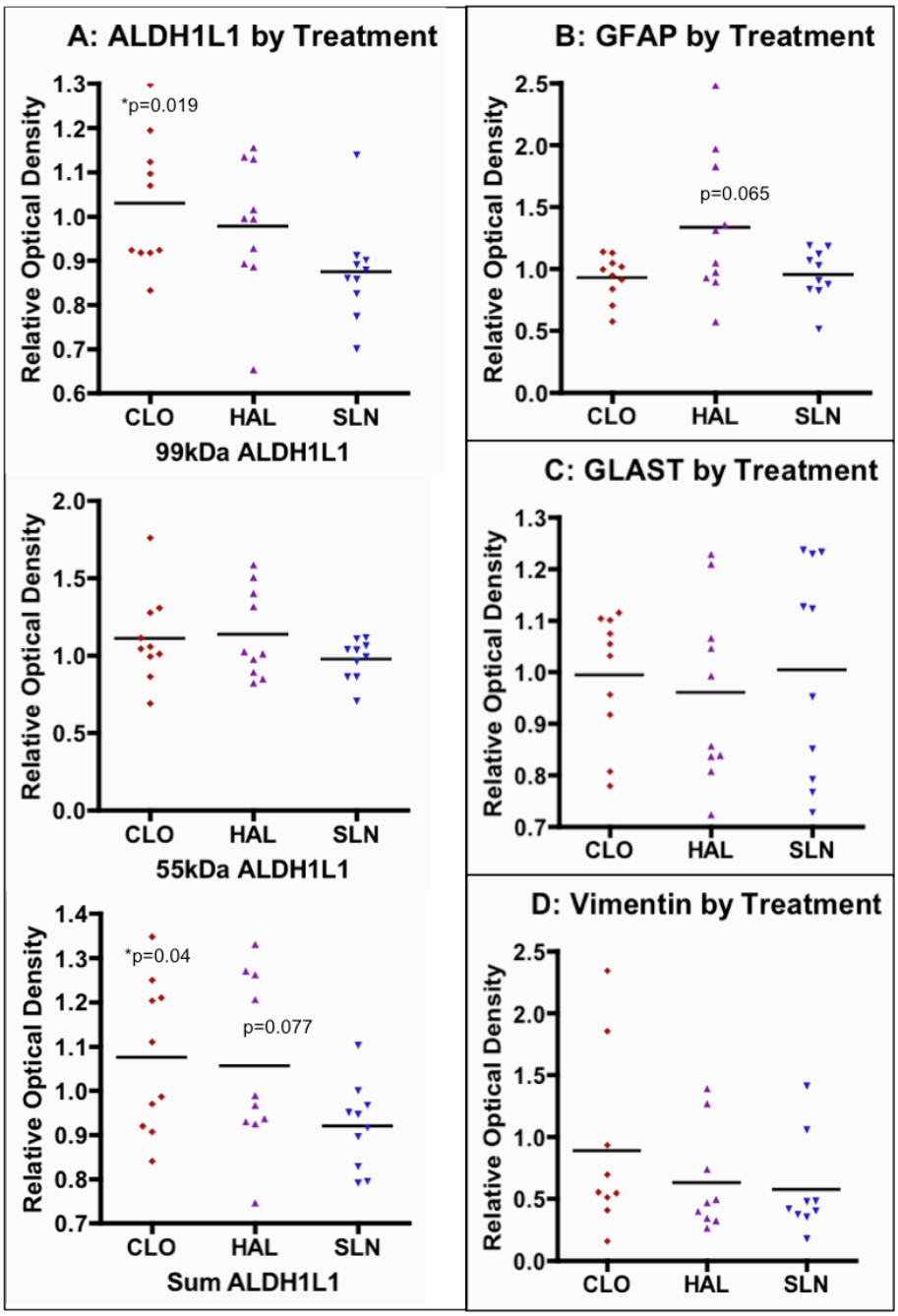

Figure 10: Antipsychotic exposure effects ALDH1L1 expression. ALDH1L1 (99kDa, 55kDa, and summed bands), GFAP, GLAST (referred to as EAAT1 in humans), and vimentin protein expression were quantified in the cingulate cortex of rats exposed to clozapine (CLO, n=10), haloperidol (HAL, n=10), or saline (SLN, n=10). While ANOVAs did not indicate that proteins differed between groups, contrast analysis revealed changes in ALDH1L1 and GFAP. Data is represented as untransformed values, with means represented by a bar. Significance values (contrast analysis, compared against the SLN group) are shown where $p<0.10$. Asterisks (*) indicate $p<0.05$.

3.3. Immunohistochemical observations of GFAP and ALDH1L1 staining in human tissue

Human tissue was treated with standard immunohistochemical procedures, and the resultant staining was qualitatively assessed with a light field microscope.

3.3.1. Regional distribution

GFAP staining was much more prominent in white matter than in grey matter (Figure 11A-C). Conversely, ALDH1L1 staining in human tissue was more evenly distributed throughout grey and white matter (Figure 11D-F).

3.3.2. Cellular distribution

In white matter, large GFAP-positive cell bodies with complex processes were observed, as was a fine network of processes surrounding them (Figure 11B; Figure 12A,B). In grey matter, few GFAP-immunoresponsive cells were stained and these were represented as small cell bodies without defined processes (Figure 11C). The strength of staining in these cell bodies varied dramatically from very strong to barely noticeable, making it difficult to identify all stained cells.

ALDH1L1 stained cell bodies relatively strongly, and cell processes more weakly. More cell processes were observable in white matter than in grey matter, though some cell processes were observed in grey matter (Figure 11F, arrow). This may be due to morphological differences between these two astrocyte populations. Based on cell morphology, we suggest that ALDH1L1 is indeed staining astrocytes (Figure 11D, inset; Figure 12C,D).

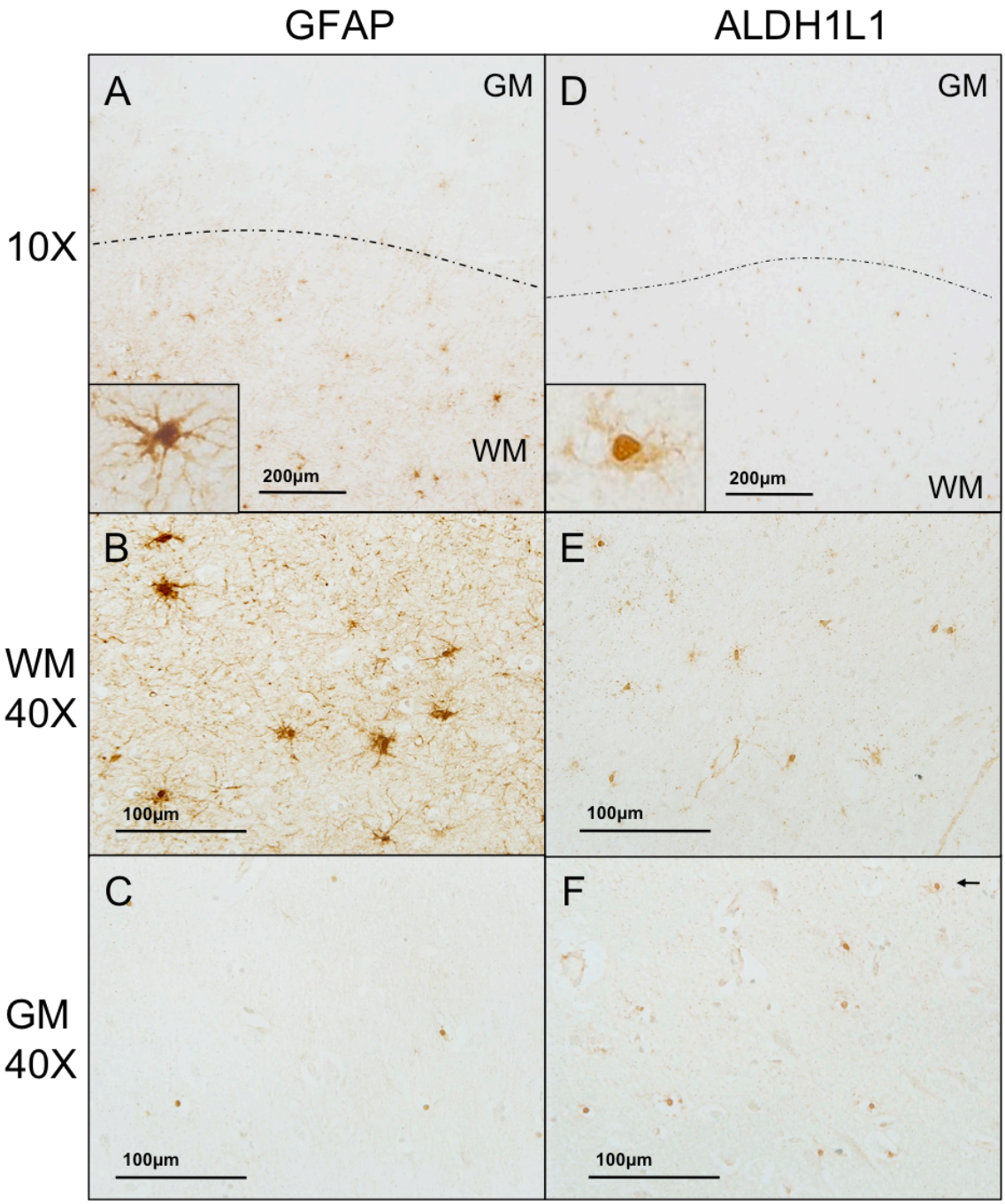

Figure 11: Anti-ALDH1L1 stains human grey and white mater more evenly than does anti-GFAP. Antibodies against GFAP (A,B,C) and ALDH1L1 (D,E,F) were used to stain human frontal cortical tissue. Cellular staining and distribution were assessed in grey matter (GM) and white matter (WM) with a light-field microscope at 10X (A,D) and 40X magnifications (A-inset, B,C,D-inset,E,F). In A and D, dashed lines indicate the approximate boundary between GM (above) and WM (below). Arrow in F emphasizes faint but present staining in cell processes in GM stained against ALDH1L1.

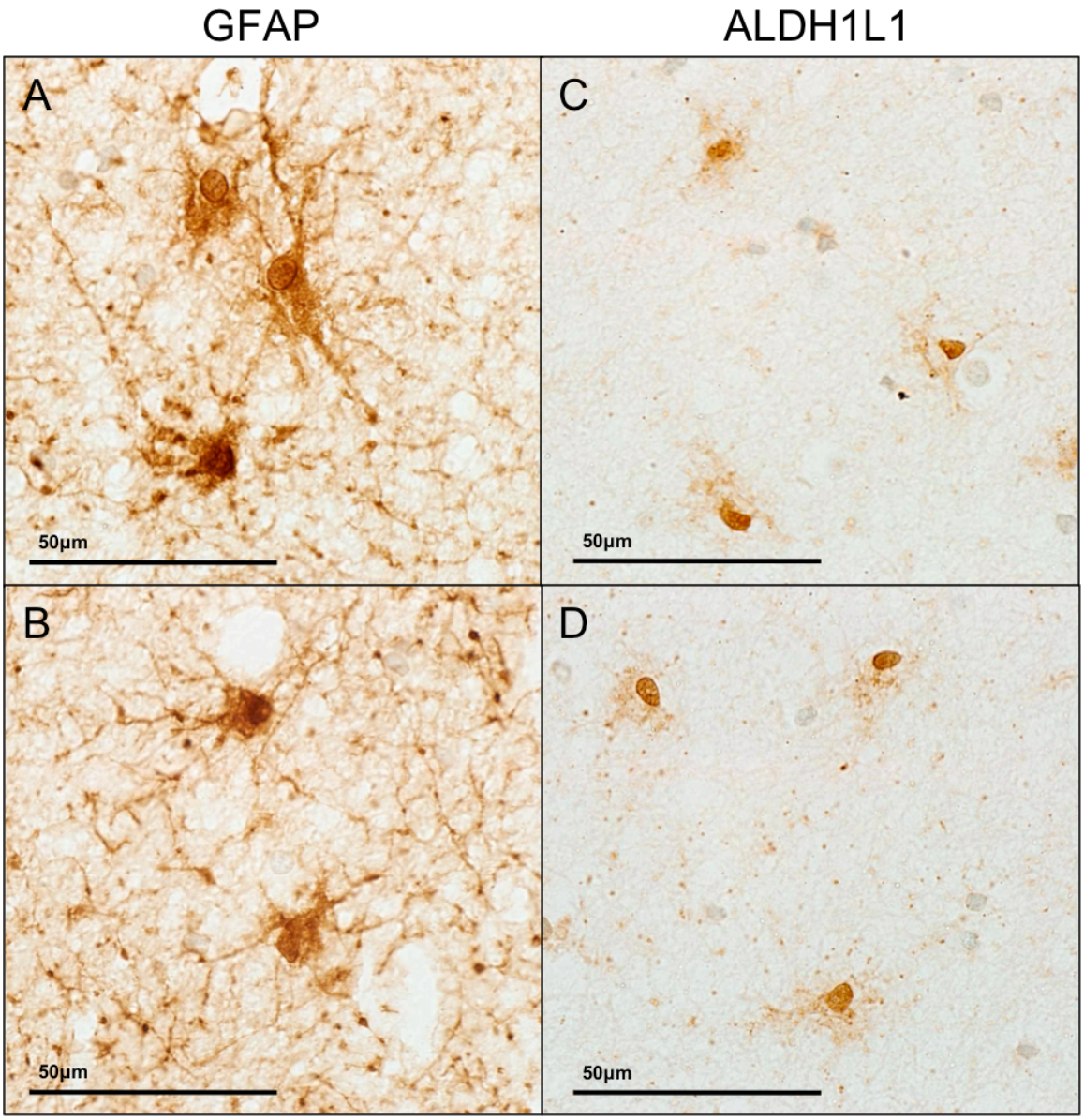

Figure 12: White matter cells stained against ALDH1L1 are morphologically similar to those stained against GFAP. Anti-GFAP (A,B) and anti-ALDH1L1 (C,D) immunohistochemical staining in human white matter at 100X magnification.

4. Discussion

We observed a significant increase in GFAP levels in SCZ (and to a lesser extent in BPD), but no change in ALDH1L1, vimentin or EAAT1 levels. While we observed trends towards increased ALDH1L1, and to a lesser extent GFAP, in antipsychotic-treated rats, we demonstrated an overall lack of significant effect of antipsychotics on astrocyte protein expression in rats.

4.1. GFAP changes in psychotic disorder

Several groups have quantified GFAP mRNA or protein levels in grey matter throughout the brain in psychotic disorders, as discussed in section 1.9, but findings are inconsistent and may be highly region-specific. These inconsistencies may be due to variations in methodology, statistical analysis, and/or samples. Overall, while data provide evidence for variability in GFAP in psychotic illness, the exact nature of this variability, and the pathological implications of these findings, both remain to be determined.

There are several possibilities that could explain why GFAP expression may be increased in the absence of changes in other astrocyte-associated proteins. First, low levels of astrocyte activation insufficient to increase vimentin expression may result in increased GFAP (Raivich et al. 1999). While vimentin expression is associated with highly activated astrocytes and glial scarring at the site of an injury **(**Pekny, 2001), GFAP is also upregulated further from injury sites, and in non-ramified astrocytes (Liberto et al. 2004). In addition, it has been asserted by several authors (Ridet et al, 1997; Raivich et al. 1999; Liberto et al. 2004; Nash et al. 2011) that astrocytes have an alternative activation

form, not unlike the reparative activation state seen in microglia during the resolution of an immune insult (Colton, 2009). Alternative, or "isomorphic," astrocyte activation is discussed in opposition to astrocyte ramification, or "anisomorphic" activation. Unlike anisomorphic activation, isomorphic activation is reversible and astrocytes in this form exhibit increased mobility and neurotrophic activity (Raivich et al. 1999; Liberto et al. 2004; Escartin et al, 2008). As astrocytes tend to ramify in culture, no protein profile of this activation form has been determined, but it is logical that vimentin expression would be less prominent or absent in this form, due to the strong association of vimentin with glial scarring (Ridet et al, 1997; Nash et al. 2011).

Second, GFAP is a cytoskeletal protein, not only providing structural support in astrocytes but also serving as a scaffolding protein, interacting with other scaffolding proteins as well as membrane proteins (Herrmann et al. 2007). A growing body of evidence supports a role for cytoskeletal pathology in SCZ and BPD (Benitez-King et al. 2004; Beasley et al., 2006; Pennington et al. 2008a; English et al. 2009; Moehle et al 2012), and it is possible that these changes could directly or indirectly affect GFAP protein levels, or be altered by them. Although we did not observe significant changes in levels of vimentin, also a cytoskeletal protein, vimentin is expressed at low to negligible levels under normal conditions. It could be argued that global abnormalities in cytoskeletal dynamics would only lead to changes in vimentin expression following astrocyte activation.

Third, it is plausible that GFAP proteins are malfunctioning in these disorders, leading to protein accumulation as a compensatory mechanism. This possibility was suggested by Rajkowska and colleagues (2002), when they observed an increase in

GFAP staining in cell bodies of Layer V PFC astrocytes, combined by a decrease in area fractionation, indicating fewer processes. This could result from genetic mutations, protein misfolding, or protein missplicing. GFAP is alternatively spliced to produce several splice variants with distinct functions, and proteomic studies have reported changes in multiple isoforms in SCZ (Johnston-Wilson et al., 2000; Pennington et al. 2008b), some of which could be splice variants. We found both the 45kDa and 50kDa isoforms of GFAP to be increased in SCZ and psychosis, but to different degrees. Further investigation of different GFAP isoforms in psychotic disease would be valuable.

Fourth, factors influencing GFAP production and/or breakdown could also be altered in psychotic disease, and may account for the changes we have observed. For example, the RNA-binding protein quaking (QKI), which is down-regulated in schizophrenia (Aberg et al. 2006; McCullumsmith et al 2007), has recently been shown to regulate GFAP mRNA expression (Radomska et al. 2013).

4.2. GFAP increases: a symptom-specific pathology?

Increases in GFAP protein content were most pronounced in the present data set when cases were divided into psychotic versus non-psychotic cohorts, suggesting that increased GFAP may be specifically associated with psychotic symptoms.

We are not the first to suggest that observed pathophysiological changes are related to the specific clinical symptom of psychosis, rather than to a diagnostic syndrome. For example, Guidotti and colleagues (2000) observed alterations in Reelin and glutamic acid decarboxylase (GAD)-67 expression in individuals with SCZ and BPD with psychosis, suggesting that these deficits may play a role in psychosis vulnerability. Olincy and

Martin (2005) observed that individuals with psychotic BPD exhibited diminished suppression of P50 auditory evoked potential like that seen in SCZ, while BPD patients without psychosis exhibited normal responses. Furthermore, several papers investigating SCZ and BPD find that BPD patients on average express changes in brain morphology and protein expression to a less marked degree than that seen in SCZ (Johnston-Wilson et al. 2000; Fatemi et al. 2000; Tkachev et al. 2003; McIntosh et al. 2005; Ellison-Wright and Bullmore, 2010). It would be interesting to see whether BPD individuals with psychosis expressed more marked differences in brain morphology and protein levels than do individuals without psychosis.

Symptom-specific neurological phenotypes could perhaps explain the breadth of protein and genetic overlap in apparently distinct psychotic disorders, as well as provide insight into other groups of disorders that share symptoms, such as mood or anxiety disorders. It is possible that each symptom of a disorder is a physiological state that may be treatable independently. Approaching psychiatric research from this angle may open up many new and exciting avenues of investigation.

4.3. No changes observed in other proteins

In the present study, we did not observe any significant diagnosis-dependent changes in expression of ALDH1L1, EAAT1, or vimentin. As each of these proteins serve different functions, negative results provide evidence for lack of change in those areas and are therefore meaningful.

ALDH1L1

ALDH1L1 is a metabolic protein recently identified as a general astrocytic marker (Cahoy et al., 2008; Yang et al., 2011). Our findings are consistent with those of Katsel and colleagues (2011), who found no change in ALDH1L1 mRNA expression in the ACC, although increased ALDH1L1 mRNA expression has been noted in several subcortical regions in SCZ (Barley et al. 2009). It is thus entirely possible that ALDH1L1 varies in psychotic disorders in other regions. Lack of change in ALDH1L1 levels in DLPFC grey matter suggests that astrocyte cell density does not differ significantly across diagnoses, at least in this region.

VIMENTIN

Vimentin is a structural protein, closely related to GFAP and expressed primarily in immature and/or highly activated astrocytes. Vimentin is considered to be a marker of astrocyte activation and gliosis (Calvo et al. 1991). Limited research has been conducted specifically on vimentin expression in psychotic disorder, however there is extensive literature supporting a lack of gliosis in SCZ and BPD (Roberts et al. 1986; 1987; Falkai et al. 1999; Rajkowska et al. 2001). While we did notice increased vimentin in individuals with a history of drug use, absence of significant group differences in vimentin expression in our study is in agreement with the mRNA findings of Katsel and colleagues (2011) in ACC grey matter.

EAAT1

EAAT1 is an astrocyte-specific glutamate transporter, and plays an important role in the astrocyte-mediated glutamate-glycine cycle (Danbolt, 2001). Previous studies of EAAT1 have produced conflicting results. Bauer and colleagues (2008) observed decreased EAAT1 protein levels in the ACC, but increased EAAT1 mRNA transcripts in the same region, suggesting translational difficulties. In a later publication, Bauer and colleagues (2010) also observed reduced EAAT1 glycosylation in the ACC in SCZ, suggesting further deficits of protein function. However, Rao and colleagues (2012) observed increased EAAT1 mRNA and protein levels in the PFC (BA 10) in SCZ and BPD. This discrepancy may be the result of differences in age (Bauer and colleagues' control group were 10 years older, on average, than their SCZ group, while Rao and colleagues' control group were 10 years younger, on average), or could be evidence of regional differences within the frontal cortex. Increased EAAT1 mRNA expression has also been observed in the thalamus in SCZ (Smith et al. 2001), while decreased EAAT1 protein expression has been observed in the temporal cortex (Shan et al. 2013) in SCZ, again suggesting that pathology-related variation in EAAT1 is region-specific. We did not find any changes in EAAT1 protein levels between groups in the DLPFC, indicating lack of change in astrocytic glutamatergic uptake, at least in this sample set.

Our results regarding EAAT1/GLAST are complicated by investigations in mice indicating that GLAST antigenicity is lost during the interval between death and preservation, with a substantial reduction in immunoreactivity associated with PMI greater than 24 hours (Li et al. 2012). In agreement with this, we observed a significant negative correlation between EAAT1 and PMI, and so included this variable as a

covariate in ANCOVA analysis**.** With PMI accounted for in this way, we failed to see significant variation in EAAT1 levels between cohorts, however when we removed samples with PMI over 24 hours from the analysis we found that EAAT1 was significantly increased in psychotic individuals. This phenomenon was also observed when individuals with low brain pH were excluded from analysis. Brain pH is closely associated with PMI, dropping with increased PMI (Stan et al. 2006).

Increased EAAT1 protein expression may indicate an increase in glutamatergic reuptake, which would coincide with a hyperglutamatergic state in the prefrontal cortex, in keeping with the neurotransmitter imbalance theory put forth by Lisman and colleagues (2008). These findings indicate the importance of considering factors such as post-translational modifications, isoforms, and protein degradation, when examining protein expression. It is also possible that the observed increase in EAAT1 expression in individuals with short PMI, or less acidic brain tissue, is associated with our observed increases in GFAP expression. Authors have observed increases in EAAT1 levels in response to cytokine exposure (Suzuki et al. 2001), as well as decreases in response to trauma (Matsuura et al. 2002; Sung et al. 2003), which would support the theory that our observed changes are due to increased isomorphic (alternative) immune activation. Furthermore, GFAP and EAAT1 interact within the cytoskeleton (Sullivan et al. 2007), so increased GFAP may in turn lead to increased EAAT1.

4.4. Effect of antipsychotic drugs on astrocyte protein levels in rats

The majority of psychotic patients in this study were prescribed antipsychotics at time of death. Because it has been reported that chronic exposure to antipsychotic

medications may decrease astrocyte numbers (Konopaske et al. 2008), we assessed the effects of haloperidol and clozapine on astrocyte specific proteins in rat cingulate cortex.

Expression levels did not differ significantly between groups, although we found trends towards increased ALDH1L1 and GFAP expression in antipsychotic treated animals. While this is not consistent with the findings of Fatemi and colleagues (2008), who reported decreased GFAP expression following 21-day exposure to 20mg/kg clozapine in frontal grey matter in rats, these authors also observed no change in GFAP expression following 21-day exposure to 1.5mg/kg haloperidol treatment. Steffek and colleagues (2008) also found no change in GFAP levels after 28 days exposure to 1mg/kg haloperidol. The discrepancy seen between our non-significant results with 28-day 20mg/kg clozapine treatment and the significant decrease observed by Fatemi and colleagues (2008) with the same dosage and decreased duration (21 days), may be due to differences in age of the rats used. Our study used adult rats weighing 270-320 g, while the rats used by Fatemi and colleagues weighed an average of 250 g, indicating a younger cohort. Furthermore, Fatemi and colleagues anesthetized their rats before decapitation with ketamine, while we did not. This may result in slightly different neural environments that could effect protein expression in astrocytes.

It could be argued that sub-chronic exposure to antipsychotics might not mimic lifetime treatment in humans, and longer-term studies in rodents may provide a better approximation of human medication use. However, Shan and colleagues (2013) also found no changes in GLAST expression in rats after nine months of chronic haloperidol exposure. In light of these results, we tentatively suggest that it is unlikely that antipsychotics fully account for the changes in GFAP seen in our human study.

To our knowledge, no previous investigations into the effects of antipsychotic treatment on ALDH1L1 expression in rats have been conducted. Because we did not observe changes in ALDH1L1 levels in our human samples, it is unlikely that the effect of treatment on ALDH1L1 expression observed in rats is indicative of any effect of medication on ALDH1L1 expression in humans. However, it is possible that antipsychotic-induced increases in ALDH1L1 protein expression in our human samples have masked illness-associated decreases. In order to test this hypothesis, drug-naïve post-mortem samples would have to be tested.

4.5. ALDH1L1 as a marker for astrocytes in grey matter

While other authors have looked at ALDH1L1 staining in murine tissue (Cahoy et al. 2008; Yang et al. 2011), the present results are the first time this staining has been compared to GFAP staining in human grey matter. As has been discussed above, GFAP staining is difficult to interpret, because the GFAP content of protoplasmic astrocytes located in grey matter is low when compared to the fibrous astrocytes in white matter. We have observed that ALDH1L1 is more evenly expressed throughout these two tissue types and, as is demonstrated in Figure 11D, stains astrocytes in grey matter at similar levels to those seen in white matter. While these findings are only preliminary, they warrant further investigation.

5. Limitations

5.1. Methodological limitations

5.1.1. Western blotting

Western blotting, though heavily used in the scientific community, is an inexact mode of measuring relative protein concentration. The use of alternative methodologies such as polymerase chain reaction (PCR) would be a fascinating avenue to pursue in the future.

5.1.2. Cellular distribution

Other authors, such as Rajkowska and colleagues, have investigated the laminar distribution of GFAP staining in these disorders. It would be very enlightening to investigate whether the laminar distribution of ALDH1L1 positive cells is altered in psychotic disorders.

5.1.3. Rat studies

We investigated the effects of antipsychotic exposure on astrocytes protein levels in rats in an effort to understand the effects these drugs have on humans. It is, however, important to remember that rats and humans are different species, and to interpret results accordingly. It could also be argued that sub-chronic exposure to antipsychotics might not mimic lifetime treatment in humans, and longer-term studies in rodents or primates may provide a better approximation of human medication use.

5.2. Working with post-mortem tissue: external variables

One of the primary concerns when studying human tissue is that the research material is not generated, collected, and preserved in a vacuum. This means that factors such as age, sex, post mortem interval, and brain pH, cannot be controlled for experimentally, and may influence protein expression. While we can account for the effects of these external factors to some extent in statistical analysis, by including them as covariates in ANCOVA analyses, this approach has limitations and complications.

First, including an external variable as a covariate in ANCOVA analysis may not sufficiently control for the effects of the external variable, such as we have seen in the case of PMI influencing EAAT1 expression. Second, it is possible that the disorder in question could be influencing the external variable, in which case eliminating the effects of that variable may mask actual effects of the disorder on protein expression. For instance, in this study brain pH was decreased in SCZ and psychosis, and negatively correlated with vimentin and GFAP expression. It has been suggested that SCZ and BPD are associated with decreased pH levels, either due to the disease process, cigarette and other substance use, or increased rate of violent death (Websters, 2006).

5.3. Sex differences

According to our analyses, much of the GFAP variability seen between groups and by psychosis seems to be carried by the female cohort (see section 3.1.6.2). Sexual dimorphism is a common finding in clinical studies of psychotic disorders, with female patients exhibiting less severe illness trajectory, with increased risk for depression and decreased negative symptoms when compared to their male counterparts (Morgan et al.

2008; Abel et al. 2010). Women also tend to exhibit fewer morphological brain changes than their male counterparts, with less hippocampal atrophy (Exner et al. 2008) or ventricular enlargement (Andreasen et al. 1990; Leung and Chue, 2000; Abel et al. 2010). However one group did observe decreased functional connectivity throughout the brain in females with SCZ when compared to males, as measured by EEG (Selwa-Younan et al. 2004), and another observed increased cortical thinning in females when compared to males with MRI (Narr et al. 2005).

Expression of GFAP has been shown to be dimorphic between sexes in rats, specifically increasing during proestrus in the hippocampus (Arias et al. 2009). Furthermore, Cordeau and colleagues (2008) observed a significant increase in GFAP upregulation in response to ischemia in female mice during metestrus and diestrus periods. This altered response was prevented with estrogen hormone therapy. These cyclical changes in GFAP expression in females suggest that female astrocytes respond to the fluctuating sex hormones of the fertility cycle.

It has been suggested that estrogen exerts a protective influence on the brain in this disorder, and its efficacy as a treatment for SCZ is currently being investigated (Begemann et al, 2012). The fact that estrogen also regulates GFAP expression might support our observation of increased GFAP in females with psychosis. It is possible that the changes we are seeing in GFAP expression could be protective and/or compensatory.

5.4. Substance abuse

It is difficult to rule out potential effects of substances such as nicotine, illicit drugs, and alcohol on protein expression. Subjects with a diagnosis of SCZ or BPD were

significantly more likely to have histories of alcohol or illicit drug use in this study, and exploratory analyses revealed potential influences of drug and alcohol use on GFAP and vimentin levels.

Vimentin levels were increased in heavy drug and alcohol users overall, although this finding was only significant in the BPD group, These data suggest that heavy alcohol/drug use may lead to astrocyte activation, and are consistent with animal studies that have reported increased vimentin expression in a binge drinking model (Hayes et al., 2013). However, as we failed to see significant effect of diagnosis on vimentin levels it is unlikely that substance use had a dramatic impact on our results overall.

We also observed increases in total GFAP levels in subjects with heavy drug use. However, when these samples were removed from the analysis, GFAP levels were still significantly increased in SCZ and in the psychotic cohort, suggesting that our findings are not wholly attributable to drug use. Combined with observed increases in vimentin levels associated with heavy drug use, we suggest that GFAP increases associated with drug use are the result of drug-induced gliosis, independent of any effects of psychotic illness. However, given the limitations of this data, such as unreliability of information obtained after death, lack of details regarding duration or type of substance used, and inexact information recording methods, these results should be interpreted with caution.

Astrocytic cell loss and damage is apparent in chronic drug users in several cortical regions (Sastre et al. 1996; Buttner and Weis, 2006), with the marked exception of the hippocampus, where increased astrogliosis was apparent with GFAP staining in fatally intoxicated drug addicts (Weber et al. 2013), as well as in a large cohort of intravenous drug users (Oehmichen et al. 1995). In rats, astrocytes respond to methamphetamine

exposure with pronounced activation, when administered *in vivo* (Fumagalli et al. 1998) or in culture (Lau et al. 2000). However chronic heroin exposure appears to precipitate astrocytic cell death (Emeterio et al. 2006) and decrease astrocyte markers (Sastre et al. 1996) in rats.

Other authors have demonstrated that chronic alcohol exposure leads to astrocyte damage. GFAP levels were significantly decreased in the frontal cortex (Lewohl et al. 2005) and hippocampus (Korbo et al. 1999) of chronic alcoholics, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) signal was increased in GFAP-responsive astrocytes in the frontal cortex and hippocampus (Ikegami et al. 2003). In rats chronically exposed to ethanol, astrocytes showed signs of marked gliosis and increased S-100B production, a neurotrophic factor expressed in astrocytes and oligodendrocytes on immune activation (Evrard et al. 2006). It is thus clear that severe alcohol and drug abuse both have profound and broad effects on astrocytes, and it is possible that our results were affected by the higher substance usage of our psychiatric cohorts.

5.5. Prescribed medication

When studying psychiatric patients, it can be very difficult to parse out the effects of treatment from the effects of the disorder. This is further complicated in the case of antipsychotic medication, where the long-term neurological effects of this medication may be associated with alleviation of symptoms, or may be the underlying cause of adverse side effects (Serretti et al. 2004).

5.5.1. Antipsychotic medications

The effects of antipsychotic medication on astrocytes remain unclear. We assessed the effects of haloperidol and clozapine in rats, and saw no significant changes in protein levels. Our results are discussed in the context of current literature in section 4.4. While we failed to observe an effect of antipsychotic dose on protein expression in our data set, the overwhelming majority of psychotic patients in this study were prescribed antipsychotics. It is thus possible that the changes observed in thus study are associated with the effects of long term antipsychotic treatment. While further investigation into astrocyte protein expression in medication-naïve patients would be valuable, this tissue is extremely difficult to come by.

5.5.2. Mood stabilizers

While we lacked complete information regarding the amount of mood stabilizers prescribed to individuals in this cohort, data indicates that 23/34 of the BPD patients were prescribed one or more mood stabilizers at time of death. It is possible that these drugs altered astrocyte function and expression of the proteins studied here.

Several authors have observed effects of mood stabilizers on astrocytes. Increases in GFAP and gliosis have been observed in the hippocampus of lithium treated rats (Rocha et al. 1998; Rocha and Rodnight, 1994), and lithium treatment was shown to enhance glutamine release by astrocytes cultured with neurons (Fan et al. 2010). Chronic valproic acid treatment appears to decrease GFAP expression in rat frontal cortex (Fatemi et al. 2008), and exposure of cultured astrocytes to valproate increased release of astrocytic neurotrophic factors (glial-derived neurotrophic factor (GDNF) and BDNF). These

results indicate that, like antipsychotics, different mood stabilizing drugs effect astrocytes differently, however more research is needed before the effects of mood stabilizers on astrocytes can be fully understood.

6. Conclusion

In summary, we found increased GFAP protein expression in DLPFC of patients with psychotic illness, indicating a role for this protein in the pathophysiology of psychosis. We did not find any changes in levels of ALDH1L1, vimentin, or EAAT1, suggesting that dysregulation is specific to GFAP. Nor did we observe a significant effect of haloperidol or clozapine exposure on astrocyte proteins in rats. We also demonstrate the ALDH1L1 appears to be a more general stain of astrocytes in human grey matter. Our results challenge the appropriateness of GFAP as a marker for assessing total astrocyte populations in grey matter; ALDH1L1 may be more suitable. These findings add to a large body of literature implicating GFAP and astrocytes in SCZ and BPD.

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Appendix 1: DSM-IV-TR Criteria for Schizophrenia and Bipolar Disorder

Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision.

Schizophrenia

Criterion A: Two or more of the following symptoms must be present for a significant portion of time during a one-month period (or less if successfully treated).

1. delusions
2. hallucinations
3. disorganized speech, such as frequent derailment or incoherence
4. grossly disorganized or catatonic behavior
5. negative symptoms, such as affective flattening, alogia, or avolition

Criterion B: A major area of function such as work, interpersonal relations or self-care is severely impacted for a significant portion of the time since the onset of the disturbance.

Criterion C: The disturbance persists continuously for at least six months. These six months must include at least one month of symptoms that meet criterion A.

Criterion D: During the period of six months described under Criterion C, there have been no major Depressive, Manic or Mixed Episodes occurring concurrently with the symptoms of Criterion A. Further, if at all any mood disorder episodes have occurred

during the six-month period, these episodes have been of a duration that is much less than the period when the Criterion A symptoms were active.

Criterion E: The disturbance is not an outcome of the physiological effects of a substance or a general medical condition.

Bipolar Disorder

BPD I: Characterized by the occurrence of one or more manic or mixed episodes

BPD II: Characterized by at least one hypomanic episode, and one or more major depressive episodes.

Manic Episode:

Criterion A: A distinct period of abnormally and persistently elevated, expansive, or irritable mood, lasting at least 1 week (or any duration if hospitalization is necessary).

Criterion B: Three (or more) of the following symptoms have persisted (four if the mood is only irritable) and have been present to a significant degree:

1. inflated self-esteem or grandiosity
2. decreased need for sleep (e.g., feels rested after only 3 hours of sleep)
3. more talkative than usual or pressure to keep talking
4. flight of ideas or subjective experience that thoughts are racing

5. distractibility (i.e., attention too easily drawn to unimportant or irrelevant external stimuli)
6. increase in goal-directed activity (either socially, at work or school, or sexually) or psychomotor agitation
7. excessive involvement in pleasurable activities that have a high potential for painful consequences (e.g., engaging in unrestrained buying sprees, sexual indiscretions, or foolish business investments)

Criterion C: The symptoms do not meet criteria for a Mixed Episode.

Criterion D: The mood disturbance is sufficiently severe to cause marked impairment in occupational functioning or in usual social activities or relationships with others, or to necessitate hospitalization to prevent harm to self or others, or there are psychotic features.

Criterion E: The symptoms are not due to the direct physiological effects of a substance or a general medical condition.

Major Depressive Episode:

Criterion A: Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood, or (b) loss of interest or pleasure.

1. depressed mood most of the day, nearly every day, as indicated by either subjective report or observation made by others
2. markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day
3. significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day
4. Insomnia or Hypersomnia nearly every day
5. psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down)
6. fatigue or loss of energy nearly every day
7. feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day
8. diminished ability to think or concentrate, or indecisiveness, nearly every day
9. recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide

Criterion B: The symptoms do not meet criteria for a Mixed Episode (see p. 335).

Criterion C: The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

Criterion D: The symptoms are not due to the direct physiological effects of a substance or a general medical condition.

Criterion E: The symptoms are not better accounted for by Bereavement.

Mixed Episode:

Criterion A: The criteria are met both for a Manic Episode and for a Major Depressive Episode (except for duration) nearly every day during at least a 1-week period.

Criterion B: The mood disturbance is sufficiently severe to cause marked impairment in occupational functioning or in usual social activities or relationships with others, or to necessitate hospitalization to prevent harm to self or others, or there are psychotic features.

Criterion C: The symptoms are not due to the direct physiological effects of a substance or a general medical condition.